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MULTI-ARMED, MONOFUNCTIONAL, AND HYDROLYTICALLY STABLE
DERIVATIVES OF
POLY(ETHYLENE GLYCOL) AND
RELATED POLYMERS FOR
MODIFICATION OF SURFACES AND MOLECULES

This application is related to and claims the benefit of the filing date of USSN 08/371,065, which was filed on January 10, 1995 and is entitled MULTI-ARMED, MONOFUNCTIONAL, AND HYDROLYTICALLY STABLE

5 DERIVATIVES OF POLY(ETHYLENE GLYCOL) AND RELATED POLYMERS FOR MODIFICATION OF SURFACES AND MOLECULES.

FIELD OF THE INVENTION

This invention relates to monofunctional derivatives of poly(ethylene glycol) and related 10 polymers and to methods for their synthesis and activation for use in modifying the characteristics of surfaces and molecules.

BACKGROUND OF THE INVENTION

Improved chemical and genetic methods have 15 made many enzymes, proteins, and other peptides and polypeptides available for use as drugs or biocatalysts having specific catalytic activity. However, limitations exist to use of these compounds.

For example, enzymes that exhibit specific 20 biocatalytic activity sometimes are less useful than they otherwise might be because of problems of low stability and solubility in organic solvents. During *in vivo* use, many proteins are cleared from circulation too rapidly. Some proteins have less water solubility

than is optimal for a therapeutic agent that circulates through the bloodstream. Some proteins give rise to immunological problems when used as therapeutic agents. Immunological problems have been reported from

5 manufactured proteins even where the compound apparently has the same basic structure as the homologous natural product. Numerous impediments to the successful use of enzymes and proteins as drugs and biocatalysts have been encountered.

10 One approach to the problems that have arisen in the use of polypeptides as drugs or biocatalysts has been to link suitable hydrophilic or amphiphilic polymer derivatives to the polypeptide to create a polymer cloud surrounding the polypeptide. If the

15 polymer derivative is soluble and stable in organic solvents, then enzyme conjugates with the polymer may acquire that solubility and stability. Biocatalysis can be extended to organic media with enzyme and polymer combinations that are soluble and stable in

20 organic solvents.

For *in vivo* use, the polymer cloud can help to protect the compound from chemical attack, to limit adverse side effects of the compound when injected into the body, and to increase the size of the compound, potentially to render useful compounds that have some medicinal benefit, but otherwise are not useful or are even harmful to an organism. For example, the polymer cloud surrounding a protein can reduce the rate of renal excretion and immunological complications and can increase resistance of the protein to proteolytic breakdown into simpler, inactive substances.

However, despite the benefits of modifying polypeptides with polymer derivatives, additional problems have arisen. These problems typically arise

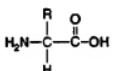
35 in the linkage of the polymer to the polypeptide. The linkage may be difficult to form. Bifunctional or multifunctional polymer derivatives tend to cross link

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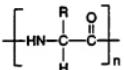
proteins, which can result in a loss of solubility in water, making a polymer-modified protein unsuitable for circulating through the blood stream of a living organism. Other polymer derivatives form

5 hydrolytically unstable linkages that are quickly destroyed on injection into the blood stream. Some linking moieties are toxic. Some linkages reduce the activity of the protein or enzyme, thereby rendering the protein or enzyme less effective.

10 The structure of the protein or enzyme dictates the location of reactive sites that form the loci for linkage with polymers. Proteins are built of various sequences of alpha-amino acids, which have the general structure



15 The alpha amino moiety ($\text{H}_2\text{N}-$) of one amino acid joins to the carboxyl moiety ($-\text{COOH}$) of an adjacent amino acid to form amide linkages, which can be represented as



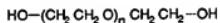
where n can be hundreds or thousands. The terminal amino acid of a protein molecule contains a free alpha 20 amino moiety that is reactive and to which a polymer can be attached. The fragment represented by R can contain reactive sites for protein biological activity and for attachment of polymer.

For example, in lysine, which is an amino acid forming part of the backbone of most proteins, a reactive amino ($-\text{NH}_2$) moiety is present in the epsilon position as well as in the alpha position. The epsilon

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-NH₂ is free for reaction under conditions of basic pH. Much of the art has been directed to developing polymer derivatives having active moieties for attachment to the epsilon -NH₂ moiety of the lysine fraction of a 5 protein. These polymer derivatives all have in common that the lysine amino acid fraction of the protein typically is modified by polymer attachment, which can be a drawback where lysine is important to protein activity.

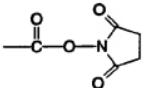
10 Poly(ethylene glycol), which is commonly referred to simply as "PEG," has been the nonpeptidic polymer most used so far for attachment to proteins. The PEG molecule typically is linear and can be represented structurally as



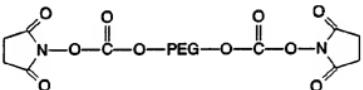
15 or, more simply, as HO-PEG-OH. As shown, the PEG molecule is difunctional, and is sometimes referred to as "PEG diol." The terminal portions of the PEG molecule are relatively nonreactive hydroxyl moieties, -OH, that can be activated, or converted to functional 20 moieties, for attachment of the PEG to other compounds at reactive sites on the compound.

For example, the terminal moieties of PEG diol have been functionalized as active carbonate ester for selective reaction with amino moieties by 25 substitution of the relatively nonreactive hydroxyl moieties, -OH, with succinimidyl active ester moieties from N-hydroxy succinimide. The succinimidyl ester moiety can be represented structurally as

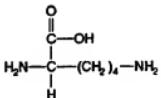
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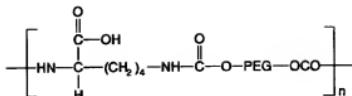
Difunctional PEG, functionalized as the succinimidyl carbonate, has a structure that can be represented as



Difunctional succinimidyl carbonate PEG has been reacted with free lysine monomer to make high 5 molecular weight polymers. Free lysine monomer, which is also known as alpha, epsilon diaminocaproic acid, has a structure with reactive alpha and epsilon amino moieties that can be represented as



These high molecular weight polymers from 10 difunctional PEG and free lysine monomer have multiple, pendant reactive carboxyl groups extending as branches from the polymer backbone that can be represented structurally as

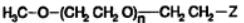


The pendant carboxyl groups typically have been used to couple nonprotein pharmaceutical agents to the polymer. Protein pharmaceutical agents would tend to be cross linked by the multifunctional polymer with 5 loss of protein activity.

Multiarmed PEGs having a reactive terminal moiety on each branch have been prepared by the polymerization of ethylene oxide onto multiple hydroxyl groups of polyols including glycerol. Coupling of this 10 type of multi-functional, branched PEG to a protein normally produces a cross-linked product with considerable loss of protein activity.

It is desirable for many applications to cap the PEG molecule on one end with an essentially 15 nonreactive end moiety so that the PEG molecule is monofunctional. Monofunctional PEGs are usually preferred for protein modification to avoid cross linking and loss of activity. One hydroxyl moiety on the terminus of the PEG diol molecule typically is 20 substituted with a nonreactive methyl end moiety, CH_3- . The opposite terminus typically is converted to a reactive end moiety that can be activated for attachment at a reactive site on a surface or a molecule such as a protein.

25 PEG molecules having a methyl end moiety are sometimes referred to as monomethoxy-poly(ethylene glycol) and are sometimes referred to simply as "mPEG." The mPEG polymer derivatives can be represented structurally as



where n typically equals from about 45 to 115 and -Z is a functional moiety that is active for selective attachment to a reactive site on a molecule or surface or is a reactive moiety that can be converted to a 5 functional moiety.

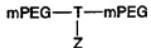
Typically, mPEG polymers are linear polymers of molecular weight in the range of from about 1,000 to 5,000. Higher molecular weights have also been examined, up to a molecular weight of about 25,000, but 10 these mPEGS typically are not of high purity and have not normally been useful in PEG and protein chemistry. In particular, these high molecular weight mPEGS typically contain significant percentages of PEG diol.

Proteins and other molecules typically have a 15 limited number and distinct type of reactive sites available for coupling, such as the epsilon -NH₂ moiety of the lysine fraction of a protein. Some of these reactive sites may be responsible for a protein's biological activity. A PEG derivative that attached to 20 a sufficient number of such sites to impart the desired characteristics can adversely affect the activity of the protein, which offsets many of the advantages otherwise to be gained.

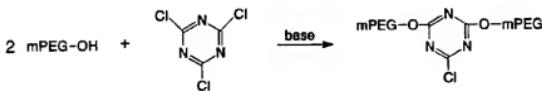
Attempts have been made to increase the 25 polymer cloud volume surrounding a protein molecule without further deactivating the protein. Some PEG derivatives have been developed that have a single functional moiety located along the polymer backbone for attachment to another molecule or surface, rather 30 than at the terminus of the polymer. Although these compounds can be considered linear, they are often referred to as "branched" and are distinguished from conventional, linear PEG derivatives since these

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molecules typically comprise a pair of mPEG- molecules that have been joined by their reactive end moieties to another moiety, which can be represented structurally as -T-, and that includes a reactive moiety, -Z, extending from the polymer backbone. These compounds have a general structure that can be represented as



These monofunctional mPEG polymer derivatives show a branched structure when linked to another compound. One such branched form of mPEG with a single 10 active binding site, -Z, has been prepared by substitution of two of the chloride atoms of trichloro-s-triazine with mPEG to make mPEG-disubstituted chlorotriazine. The third chloride is used to bind to protein. An mPEG disubstituted 15 chlorotriazine and its synthesis are disclosed in Wada, H., Imamura, I., Sako, M., Katagiri, S., Tarui, S., Nishimura, H., and Inada, Y. (1990) Antitumor enzymes: polyethylene glycol-modified asparaginase. Ann. N.Y. Acad. Sci. 613, 95-108. Synthesis of mPEG 20 disubstituted chlorotriazine is represented structurally below.



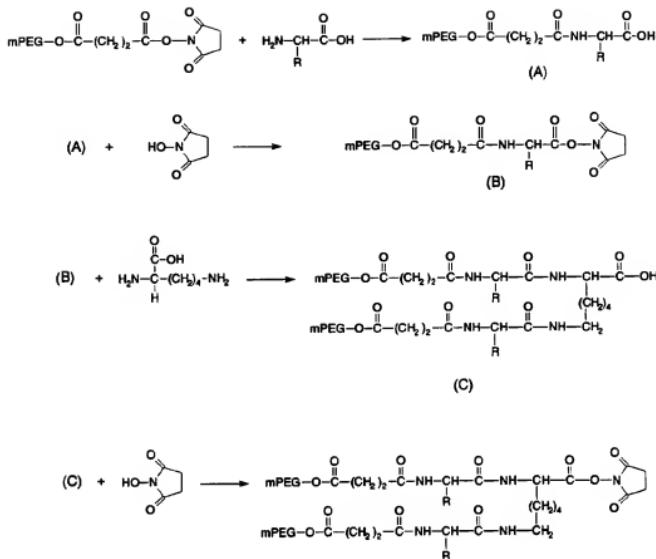
However, mPEG-disubstituted chlorotriazine and the procedure used to prepare it present severe 25 limitations because coupling to protein is highly nonselective. Several types of amino acids other than

lysine are attacked and many proteins are inactivated. The intermediate is toxic. Also, the mPEG-disubstituted chlorotriazine molecule reacts with water, thus substantially precluding purification of 5 the branched mPEG structure by commonly used chromatographic techniques in water.

A branched mPEG with a single activation site based on coupling of mPEG to a substituted benzene ring is disclosed in European Patent Application Publication 10 No. 473 084 A2. However, this structure contains a benzene ring that could have toxic effects if the structure is destroyed in a living organism.

Another branched mPEG with a single activation site has been prepared through a complex 15 synthesis in which an active succinate moiety is attached to the mPEG through a weak ester linkage that is susceptible to hydrolysis. An mPEG-OH is reacted with succinic anhydride to make the succinate. The reactive succinate is then activated as the 20 succinimide. The synthesis, starting with the active succinimide, includes the following steps, represented structurally below.

- 10 -



The mPEG activated as the succinimide, mPEG succinimidl succinate, is reacted in the first step as shown above with norleucine. The symbol -R in the synthesis represents the n-butyl moiety of norleucine.

5 The mPEG and norleucine conjugate (A) is activated as the succinimide in the second step by reaction with N-hydroxy succinimide. As represented in the third step, the mPEG and norleucine conjugate activated as the succinimide (B) is coupled to the alpha and epsilon 10 amino moieties of lysine to create an mPEG disubstituted lysine (C) having a reactive carboxyl moiety. In the fourth step, the mPEG disubstituted lysine is activated as the succinimide.

The ester linkage formed from the reaction of 15 the mPEG-OH and succinic anhydride molecules is a weak linkage that is hydrolytically unstable. In vivo

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application is therefore limited. Also, purification of the branched mPEG is precluded by commonly used chromatographic techniques in water, which normally would destroy the molecule.

5 The molecule also has relatively large molecular fragments between the carboxyl group activated as the succinimide and the mPEG moieties due to the number of steps in the synthesis and to the number of compounds used to create the fragments.

10 These molecular fragments are sometimes referred to as "linkers" or "spacer arms," and have the potential to act as antigenic sites promoting the formation of antibodies upon injection and initiating an undesirable immunological response in a living organism.

15 SUMMARY OF THE INVENTION

The invention provides a branched or "multi-armed" amphiphilic polymer derivative that is monofunctional, hydrolytically stable, can be prepared in a simple, one-step reaction, and possesses no aromatic moieties in the linker fragments forming the linkages with the polymer moieties. The derivative can be prepared without any toxic linkage or potentially toxic fragments. Relatively pure polymer molecules of high molecular weight can be created. The polymer can be purified by chromatography in water. A multi-step method can be used if it is desired to have polymer arms that differ in molecular weight. The polymer arms are capped with relatively nonreactive end groups. The derivative can include a single reactive site that is located along the polymer backbone rather than on the terminal portions of the polymer moieties. The reactive site can be activated for selective reactions.

The multi-armed polymer derivative of the invention having a single reactive site can be used for, among other things, protein modification with a high retention of protein activity. Protein and enzyme activity can be preserved and in some cases is

enhanced. The single reactive site can be converted to a functional group for highly selective coupling to proteins, enzymes, and surfaces. A larger, more dense polymer cloud can be created surrounding a biomolecule 5 with fewer attachment points to the biomolecule as compared to conventional polymer derivatives having terminal functional groups. Hydrolytically weak ester linkages can be avoided. Potentially harmful or toxic products of hydrolysis can be avoided. Large linker 10 fragments can be avoided so as to avoid an antigenic response in living organisms. Cross linking is avoided.

The molecules of the invention can be represented structurally as poly_a-P-CR(-Q-poly_b)-Z or:



15 Poly_a and poly_b represent nonpeptidic and substantially nonreactive water soluble polymeric arms that may be the same or different. C represents carbon. P and Q represent linkage fragments that may be the same or different and that join polymer arms 20 poly_a and poly_b, respectively, to C by hydrolytically stable linkages in the absence of aromatic rings in the linkage fragments. R is a moiety selected from the group consisting of H, substantially nonreactive, usually alkyl, moieties, and linkage fragments attached 25 by a hydrolytically stable linkage in the absence of aromatic rings to a nonpeptidic and substantially nonreactive water soluble polymeric arm. The moiety -Z comprises a moiety selected from the group consisting of moieties having a single site reactive toward 30 nucleophilic moieties, sites that can be converted to sites reactive toward nucleophilic moieties, and the reaction product of a nucleophilic moiety and moieties

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having a single site reactive toward nucleophilic moieties.

Typically, the moiety -P-CR(-Q)-Z is the reaction product of a linker moiety and the reactive 5 site of monofunctional, nonpeptidic polymer derivatives, poly_a-W and poly_b-W, in which W is the reactive site. Polymer arms poly_a and poly_b are nonpeptidic polymers and can be selected from polymers that have a single reactive moiety that can be 10 activated for hydrolytically stable coupling to a suitable linker moiety. The linker has the general structure X-CR-(Y)-Z, in which X and Y represent fragments that contain reactive sites for coupling to the polymer reactive site W to form linkage fragments P 15 and Q, respectively.

In one embodiment, at least one of the polymer arms is a poly(ethylene glycol) moiety capped with an essentially nonreactive end group, such as a monomethoxy-poly(ethylene glycol) moiety

20 ("mPEG-"), which is capped with a methyl end group, CH₃- . The other branch can also be an mPEG moiety of the same or different molecular weight, another poly(ethylene glycol) moiety that is capped with an essentially nonreactive end group other than methyl, or 25 a different nonpeptidic polymer moiety that is capped with a nonreactive end group such as a capped poly(alkylene oxide), a poly(oxyethylated polyol), a poly(olefinic alcohol), or others.

For example, in one embodiment poly_a and poly_b, 30 are each monomethoxy-poly(ethylene glycol) ("mPEG") of the same or different molecular weight. The mPEG-disubstituted derivative has the general structure mPEG_a-P-CH(-Q-mPEG_b)-Z. The moieties mPEG_a- and mPEG_b- have the structure CH₃- (CH₂CH₂O)_nCH₂CH₂- and n may be the 35 same or different for mPEG_a and mPEG_b. Molecules having values of n of from 1 to about 1,150 are contemplated.

The linker fragments P and Q contain hydrolytically stable linkages that may be the same or different depending upon the functional moiety on the mPEG molecules and the molecular structure of the 5 linker moiety used to join the mPEG moieties in the method for synthesizing the multi-armed structure. The linker fragments typically are alkyl fragments containing amino or thiol residues forming a linkage with the residue of the functional moiety of the 10 polymer. Depending on the degree of substitution desired, linker fragments P and Q can include reactive sites for joining additional monofunctional nonpeptidic polymers to the multi-armed structure.

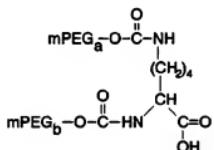
The moiety -R can be a hydrogen atom, H, a 15 nonreactive fragment, or, depending on the degree of substitution desired, R can include reactive sites for joining additional monofunctional nonpeptidic polymers to the multi-armed structure.

The moiety -Z can include a reactive moiety 20 for which the activated nonpeptidic polymers are not selective and that can be subsequently activated for attachment of the derivative to enzymes, other proteins, nucleotides, lipids, liposomes, other molecules, solids, particles, or surfaces. The moiety 25 -Z can include a linkage fragment -R_z. Depending on the degree of substitution desired, the R_z fragment can include reactive sites for joining additional monofunctional nonpeptidic polymers to the multi-armed structure.

30 Typically, the -Z moiety includes terminal functional moieties for providing linkages to reactive sites on proteins, enzymes, nucleotides, lipids, liposomes, and other materials. The moiety -Z is intended to have a broad interpretation and to include 35 the reactive moiety of monofunctional polymer derivatives of the invention, activated derivatives, and conjugates of the derivatives with polypeptides and

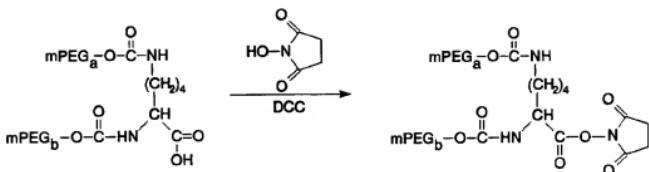
other substances. The invention includes biologically active conjugates comprising a biomolecule, which is a biologically active molecule, such as a protein or enzyme, linked through an activated moiety to the 5 branched polymer derivative of the invention. The invention includes biomaterials comprising a solid such as a surface or particle linked through an activated moiety to the polymer derivatives of the invention.

In one embodiment, the polymer moiety is an 10 mPEG moiety and the polymer derivative is a two-armed mPEG derivative based upon hydrolytically stable coupling of mPEG to lysine. The mPEG moieties are represented structurally as $\text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CH}_2-$ wherein n may be the same or different for poly_a- and poly_b- and 15 can be from 1 to about 1,150 to provide molecular weights of from about 100 to 100,000. The -R moiety is hydrogen. The -Z moiety is a reactive carboxyl moiety. The molecule is represented structurally as follows:



The reactive carboxyl moiety of 20 hydrolytically stable mPEG-disubstituted lysine, which can also be called alpha, epsilon-mPEG lysine, provides a site for interacting with ion exchange chromatography media and thus provides a mechanism for purifying the product. These purifiable, high molecular weight, 25 monofunctional compounds have many uses. For example, mPEG-disubstituted lysine, activated as succinimidyl ester, reacts with amino groups in enzymes under mild aqueous conditions that are compatible with the stability of most enzymes. The mPEG-disubstituted

lysine of the invention, activated as the succinimidyl ester, is represented as follows:



The invention includes methods of synthesizing the polymers of the invention. The 5 methods comprise reacting an active suitable polymer having the structure poly-W with a linker moiety having the structure X-CR-(Y)Z to form poly_a-P-CR(-Q-poly_b)-Z. The poly moiety in the structure poly-W can be either poly_a or poly_b and is a polymer having a single reactive 10 moiety W. The W moiety is an active moiety that is linked to the polymer moiety directly or through a hydrolytically stable linkage. The moieties X and Y in the structure X-CR-(Y)Z are reactive with W to form the linkage fragments Q and P, respectively. If the moiety 15 R includes reactive sites similar to those of X and Y, then R can also be modified with a poly-W, in which the poly can be the same as or different from poly_a or poly_b. The moiety Z normally does not include a site that is reactive with W. However, X, Y, R, and Z can 20 each include one or more such reactive sites for preparing monofunctional polymer derivatives having more than two branches.

The method of the invention typically can be accomplished in one or two steps. The method can 25 include additional steps for preparing the compound poly-W and for converting a reactive Z moiety to a functional group for highly selective reactions.

The active Z moiety includes a reactive moiety that is not reactive with W and can be activated

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subsequent to formation of poly_a-P-CR(-Q-poly_b)-Z for highly selective coupling to selected reactive moieties of enzymes and other proteins or surfaces or any molecule having a reactive nucleophilic moiety for

5 which it is desired to modify the characteristics of the molecule.

In additional embodiments, the invention provides a multi-armed mPEG derivative for which preparation is simple and straightforward.

10 Intermediates are water stable and thus can be carefully purified by standard aqueous chromatographic techniques. Chlorotriazine activated groups are avoided and more highly selective functional groups are used for enhanced selectivity of attachment and much
15 less loss of activity upon coupling of the mPEG derivatives of the invention to proteins, enzymes, and other peptides. Large spacer arms between the coupled polymer and protein are avoided to avoid introducing possible antigenic sites. Toxic groups, including
20 triazine, are avoided. The polymer backbone contains no hydrolytically weak ester linkages that could break down during in vivo applications. Monofunctional polymers of double the molecular weight as compared to the individual mPEG moieties can be provided, with mPEG
25 dimer structures having molecular weights of up to at least about 50,000, thus avoiding the common problem of difunctional impurities present in conventional, linear mPEGS.

BRIEF DESCRIPTION OF THE DRAWINGS

30 Figures 1(a), 1(b), and 1(c) illustrate the time course of digestion of ribonuclease (●), conventional, linear mPEG-modified ribonuclease (○), and ribonuclease modified with a multi-armed mPEG of the invention (■) as assessed by enzyme activity upon
35 incubation with pronase (Figure 1(a)), elastase (Figure 1(b)), and subtilisin (Figure 1(c)).

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Figures 2(a) and 2(b) illustrate stability toward heat (Figure 2(a)) and pH (Figure 2(b)) of ribonuclease (●), linear mPEG-modified ribonuclease (○), and ribonuclease modified with a multi-armed mPEG 5 of the invention (□). Figure 2(a) is based on data taken after a 15 minute incubation period at the indicated temperatures. Figure 2(b) is based on data taken over a 20 hour period at different pH values.

Figures 3(a) and 3(b) illustrate the time 10 course of digestion for catalase (●), linear mPEG-modified catalase (○), and catalase modified with a multi-armed mPEG of the invention (■) as assessed by enzyme activity upon incubation with pronase (Figure 3(a)) and trypsin (Figure 3(b)).

15 Figure 4 illustrates the stability of catalase (●), linear mPEG-modified catalase (□), and catalase modified with a multi-armed mPEG of the invention (○) for 20 hours incubation at the indicated pH values.

20 Figure 5 illustrates the time course of digestion of asparaginase (●), linear mPEG-modified asparaginase (○), and asparaginase modified with a multi-armed mPEG of the invention (■) as assessed by enzyme activity assay upon trypsin incubation.

25 Figure 6 illustrates the time course of autolysis of trypsin (●), linear mPEG-modified trypsin (■), and trypsin modified with a multi-armed mPEG of the invention (▲) evaluated as residual activity towards TAME (alpha N-p-tosyl-arginine methyl ester).

30 DETAILED DESCRIPTION

I. *Preparation of a Hydrolytically Stable mPEG-Disubstituted Lysine.*

Two procedures are described for the preparation of a hydrolytically stable, two-armed, 35 mPEG-disubstituted lysine. The first procedure is a two step procedure, meaning that the lysine is

substituted with each of the two mPEG moieties in separate reaction steps. Monomethoxy-poly(ethylene glycol) arms of different lengths or of the same length can be substituted onto the lysine molecule, if 5 desired, using the two step procedure. The second procedure is a one step procedure in which the lysine molecule is substituted with each of the two mPEG moieties in a single reaction step. The one step procedure is suitable for preparing mPEG-disubstituted 10 lysine having mPEG moieties of the same length.

Unlike prior multisubstituted structures, no aromatic ring is present in the linkage joining the nonpeptidic polymer arms produced by either the one or two step methods described below that could result in 15 toxicity if the molecule breaks down in vivo. No hydrolytically weak ester linkages are present in the linkage. Lengthy linkage chains that could promote an antigenic response are avoided.

The terms "group," "functional group," 20 "moiety," "active moiety," "reactive site," "radical," and similar terms are somewhat synonymous in the chemical arts and are used in the art and herein to refer to distinct, definable portions or units of a molecule or fragment of a molecule. "Reactive site," 25 "functional group," and "active moiety" refer to units that perform some function or have a chemical activity and are reactive with other molecules or portions of molecules. In this sense a protein or a protein residue can be considered as a molecule and as a 30 functional moiety when coupled to a polymer. A polymer, such as mPEG-COOH has a reactive site, the carboxyl moiety, -COOH, that can be converted to a functional group for selective reactions and attachment to proteins and linker moieties. The converted polymer 35 is said to be activated and to have an active moiety, while the -COOH group is relatively nonreactive in comparison to an active moiety.

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The term "nonreactive" is used herein primarily to refer to a moiety that does not readily react chemically with other moieties, such as the methyl alkyl moiety. However, the term "nonreactive" 5 should be understood to exclude carboxyl and hydroxyl moieties, which, although relatively nonreactive, can be converted to functional groups that are of selective reactivity.

The term "biologically active" means a 10 substance, such as a protein, lipid, or nucleotide that has some activity or function in a living organism or in a substance taken from a living organism. For example, an enzyme can catalyze chemical reactions.

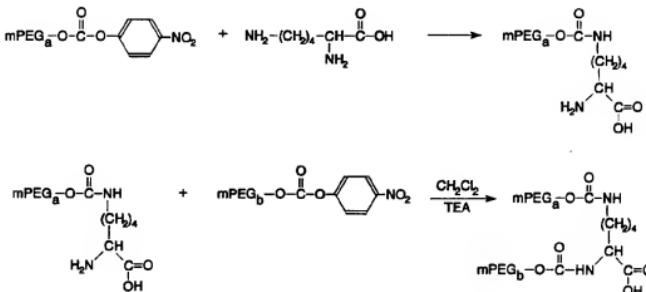
The term "biomaterial" is somewhat imprecise, and is 15 used herein to refer to a solid material or particle or surface that is compatible with living organisms or tissue or fluids. For example, surfaces that contact blood, whether *in vitro* or *in vivo*, can be made nonfouling by attachment of the polymer derivatives of 20 the invention so that proteins do not become attached to the surface.

A. Two Step Procedure

For the two step procedure, an activated mPEG is prepared for coupling to free lysine monomer and 25 then the lysine monomer is disubstituted with the activated mPEG in two steps. The first step occurs in aqueous buffer. The second step occurs in dry methylene chloride. The active moiety of the mPEG for coupling to the lysine monomer can be selected from a 30 number of activating moieties having leaving moieties that are reactive with the amino moieties of lysine monomer. A commercially available activated mPEG, mPEG-*p*-nitrophenylcarbonate, the preparation of which is discussed below, was used to exemplify the two step 35 procedure.

The two step procedure can be represented structurally as follows:

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Step 1. Preparation of mPEG-monosubstituted lysine. Modification of a single lysine amino group was accomplished with mPEG-*p*-nitrophenylcarbonate in aqueous solution where both lysine and mPEG-*p*-nitrophenylcarbonate are soluble. The mPEG-*p*-nitrophenylcarbonate has only limited stability in aqueous solution. However, lysine is not soluble in organic solvents in which the activated mPEG is stable. Consequently, only one lysine amino group is modified by this procedure. NMR confirms that the epsilon amino group is modified. Nevertheless, the procedure allows ready chloroform extraction of mPEG-monosubstituted lysine from unreacted lysine and other water soluble by-products, and so the procedure provides a desirable monosubstituted product for disubstitution.

To prepare the mPEG-monosubstituted lysine, 353 milligrams of lysine, which is about 2.5 millimoles, was dissolved in 20 milliliters of water at a pH of about 8.0 to 8.3. Five grams of mPEG-*p*-nitrophenylcarbonate of molecular weight 5,000, which is about 1 millimole, was added in portions over 3 hours. The pH was maintained at 8.3 with 0.2 N NaOH. The reaction mixture was stirred overnight at room temperature. Thereafter, the reaction mixture was cooled to 0°C and brought to a pH of about 3 with 2 N

HCl. Impurities were extracted with diethyl ether. The mPEG monosubstituted lysine, having the mPEG substituted at the epsilon amino group of lysine as confirmed by NMR analysis, was extracted three times 5 with chloroform. The solution was dried. After concentration, the solution was added drop by drop to diethyl ether to form a precipitate. The precipitate was collected and then crystallized from absolute ethanol. The percentage of modified amino groups was 10 53%, calculated by colorimetric analysis.

Step 2. Preparation of mPEG-Disubstituted Lysine. The mPEG-monosubstituted lysine product from step 1 above is soluble in organic solvents and so modification of the second lysine amino moiety can be 15 achieved by reaction in dry methylene chloride. Activated mPEG, mPEG-*p*-nitrophenylcarbonate, is soluble and stable in organic solvents and can be used to modify the second lysine amino moiety.

Triethylamine ("TEA") was added to 4.5 grams 20 of mPEG-monosubstituted lysine, which is about 0.86 millimoles. The mixture of TEA and mPEG- monosubstituted lysine was dissolved in 10 milliliters of anhydrous methylene chloride to reach a pH of 8.0. Four and nine tenths grams of mPEG-*p*- 25 nitrophenylcarbonate of molecular weight 5,000, which is 1.056 millimoles, was added over 3 hours to the solution. If it is desirable to make an mPEG disubstituted compound having mPEG arms of different lengths, then a different molecular weight mPEG could 30 have been used. The pH was maintained at 8.0 with TEA. The reaction mixture was refluxed for 72 hours, brought to room temperature, concentrated, filtered, precipitated with diethyl ether and then crystallized in a minimum amount of hot ethanol. The excess of 35 activated mPEG, mPEG-*p*-nitrophenylcarbonate, was deactivated by hydrolysis in an alkaline aqueous medium by stirring overnight at room temperature. The

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solution was cooled to 0°C and brought to a pH of about 3 with 2 N HCl.

p-Nitrophenol was removed by extraction with diethyl ether. Monomethyl-poly(ethylene glycol)-5 disubstituted lysine and remaining traces of mPEG were extracted from the mixture three times with chloroform, dried, concentrated, precipitated with diethyl ether and crystallized from ethanol. No unreacted lysine amino groups remained in the polymer mixture as 10 assessed by colorimetric analysis.

Purification of mPEG-disubstituted lysine and removal of mPEG were accomplished by gel filtration chromatography using a Bio Gel P100 (Bio-Rad) column. The column measured 5 centimeters by 50 centimeters. 15 The eluent was water. Fractions of 10 milliliters were collected. Up to 200 milligrams of material could be purified for each run. The fractions corresponding to mPEG-disubstituted lysine were revealed by iodine reaction. These fractions were pooled, concentrated, 20 and then dissolved in ethanol and concentrated. The mPEG-disubstituted lysine product was dissolved in methylene chloride, precipitated with diethyl ether, and crystallized from ethanol.

The mPEG-disubstituted lysine was also 25 separated from unmodified mPEG-OH and purified by an alternative method. Ion exchange chromatography was performed on a QAE Sephadex A50 column (Pharmacia) that measured 5 centimeters by 80 centimeters. An 8.3 mM borate buffer of pH 8.9 was used. This alternative 30 procedure permitted fractionation of a greater amount of material per run than the other method above described (up to four grams for each run).

For both methods of purification, purified mPEG-disubstituted lysine of molecular weight 10,000, 35 titrated with NaOH, showed that 100% of the carboxyl groups were free carboxyl groups. These results

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indicate that the reaction was complete and the product pure.

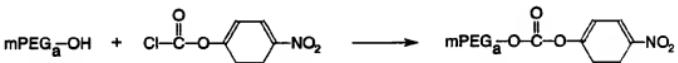
The purified mPEG-disubstituted lysine was also characterized by ¹H-NMR on a 200 MHz Bruker 5 instrument in dimethyl sulfoxide, d6, at a 5% weight to volume concentration. The data confirmed the expected molecular weight of 10,000 for the polymer. The 10 chemical shifts and assignments of the protons in the mPEG-disubstituted lysine are as follows: 1.2-1.4 ppm (multiplet, 6H, methylenes 3,4,5 of lysine); 1.6 ppm (multiplet, 2H, methylene 6 of lysine); 3.14 ppm (s, 3H, terminal mPEG methoxy); 3.49 ppm (s, mPEG backbone methylene); 4.05 ppm (t, 2H, -CH₂, -OCO-); 7.18 ppm (t, 1H, -NH- lysine); and 7.49 ppm (d, 1 H, -NH- lysine).

15 The above signals are consistent with the reported structure since two different carbamate NH protons are present. The first carbamate NH proton (at 7.18 ppm) shows a triplet for coupling with the adjacent methylene group. The second carbamate NH 20 proton (at 7.49 ppm) shows a doublet because of coupling with the α -CH of lysine. The intensity of these signals relative to the mPEG methylene peak is consistent with the 1:1 ratio between the two amide groups and the expected molecular weight of 10,000 for 25 the polymer.

30 The two step procedure described above allows polymers of different types and different lengths to be linked with a single reactive site between them. The polymer can be designed to provide a polymer cloud of custom shape for a particular application.

35 The commercially available activated mPEG, mPEG-p-nitrophenylcarbonate, is available from Shearwater Polymers, Inc. in Huntsville, Alabama. This compound was prepared by the following procedure, which can be represented structurally as follows:

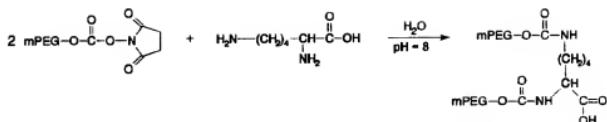
-25-



Five grams of mPEG-OH of molecular weight 5,000, or 1 millimole, were dissolved in 120 milliliters of toluene and dried azeotropically for 3 hours. The solution was cooled to room temperature and 5 concentrated under vacuum. Reactants added to the concentrated solution under stirring at 0°C were 20 milliliters of anhydrous methylene chloride and 0.4 g of *p*-nitrophenylchloroformate, which is 2 millimoles. The pH of the reaction mixture was maintained at 8 by adding 0.28 milliliters of triethylamine ("TEA"), which is 2 millimoles. The reaction mixture was allowed to stand overnight at room temperature. Thereafter, the reaction mixture was concentrated under vacuum to about 10 milliliters, filtered, and dropped into 100 15 milliliters of stirred diethyl ether. A precipitate was collected from the diethyl ether by filtration and crystallized twice from ethyl acetate. Activation of mPEG was determined to be 98%. Activation was calculated spectrophotometrically on the basis of the 20 absorption at 400 nm in alkaline media after 15 minutes of released 4-nitrophenol (ϵ of *p*-nitrophenol at 400 nm equals 17,000).

B. One Step Procedure

In the one step procedure, mPEG disubstituted 25 lysine is prepared from lysine and an activated mPEG in a single step as represented structurally below:



Except for molecular weight attributable to a longer PEG backbone in the activated mPEG used in the steps below, the mPEG disubstituted lysine of the one step procedure does not differ structurally from the 5 mPEG disubstituted lysine of the two step procedure. It should be recognized that the identical compound, having the same molecular weight, can be prepared by either method.

Preparation of mPEG disubstituted lysine by 10 the one step procedure proceeded as follows: Succinimidylcarbonate mPEG of molecular weight about 20,000 was added in an amount of 10.8 grams, which is 5.4×10^{-4} moles, to 40 milliliters of lysine HCl solution. The lysine HCl solution was in a borate 15 buffer of pH 8.0. The concentration was 0.826 milligrams succinimidylcarbonate mPEG per milliliter of lysine HCl solution, which is 1.76×10^{-4} moles. Twenty milliliters of the same buffer was added. The solution pH was maintained at 8.0 with aqueous NaOH solution for 20 the following 8 hours. The reaction mixture was stirred at room temperature for 24 hours.

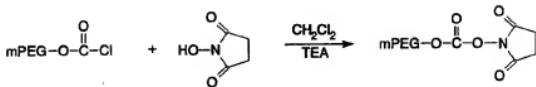
Thereafter, the solution was diluted with 300 milliliters of deionized water. The pH of the solution was adjusted to 3.0 by the addition of oxalic acid. 25 The solution was then extracted three times with dichloromethane. The combined dichloromethane extracts were dried with anhydrous sodium sulphate and filtered. The filtrate was concentrated to about 30 milliliters. The product, an impure mPEG disubstituted lysine, was

precipitated with about 200 milliliters of cold ethyl ether. The yield was 90%.

Nine grams of the above impure mPEG-disubstituted lysine reaction product was dissolved in 5 4 liters of distilled water and then loaded onto a column of DEAE Sepharose FF, which is 500 milliliters of gel equilibrated with 1500 milliliters of boric acid in a 0.5% sodium hydroxide buffer at a pH of 7.0. The loaded system was then washed with water. Impurities 10 of succinimidylcarbonate mPEG and mPEG-monosubstituted lysine, both of molecular weight about 20,000, were washed off the column. However, the desired mPEG disubstituted lysine of molecular weight 20,000 was eluted with 10 mM NaCl. The pH of the eluate was 15 adjusted to 3.0 with oxalic acid and then mPEG disubstituted lysine was extracted with dichloromethane, dried with sodium sulphate, concentrated, and precipitated with ethyl ether. Five and one tenth grams of purified mPEG disubstituted 20 lysine were obtained. The molecular weight was determined to be 38,000 by gel filtration chromatography and 36,700 by potentiometric titration.

The one step procedure is simple in application and is useful for producing high molecular 25 weight dimers that have polymers of the same type and length linked with a single reactive site between them.

Additional steps are represented below for preparing succinimidylcarbonate mPEG for disubstitution of lysine.



Succinimidylcarbonate mPEG was prepared by dissolving 30 grams of mPEG-OH of molecular weight 20,000, which is about 1.5 millimoles, in 120 milliliters of toluene. The solution was dried 5 azeotropically for 3 hours. The dried solution was cooled to room temperature. Added to the cooled and dried solution were 20 milliliters of anhydrous dichloromethane and 2.33 milliliters of a 20% solution of phosgene in toluene. The solution was stirred 10 continuously for a minimum of 16 hours under a hood due to the highly toxic fumes.

After distillation of excess phosgene and solvent, the remaining syrup, which contained mPEG chlorocarbonate, was dissolved in 100 milliliters of 15 anhydrous dichloromethane, as represented above. To this solution was added 3 millimoles of triethylamine and 3 millimoles of N-hydroxysuccinimide. The reaction mixture remained standing at room temperature for 24 hours. Thereafter, the solution was filtered through a 20 silica gel bed of pore size 60 Angstroms that had been wetted with dichloromethane. The filtrate was concentrated to 70 milliliters. Succinimidylcarbonate mPEG of molecular weight about 20,000 was precipitated in ethyl ether and dried in vacuum for a minimum of 8 25 hours. The yield was 90%. Succinimidylcarbonate-mPEG is available commercially from Shearwater Polymers in Huntsville, Alabama.

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The mPEG disubstituted lysine of the invention can be represented structurally more generally as poly_a-P-CR(-Q-poly_b)-Z or:



5 For the mPEG disubstituted lysines described above, -P-CR(-Q)-Z is the reaction product of a precursor linker moiety having two reactive amino groups and active monofunctional precursors of poly_a and poly_b that have been joined to the linker moiety at the 10 reactive amino sites. Linker fragments Q and P contain carbamate linkages formed by joining the amino containing portions of the lysine molecule with the functional group with which the mPEG was substituted. The linker fragments are selected from -O-C(O)NH(CH₂)₄- 15 and -O-C(O)NH- and are different in the exemplified polymer derivative. However, it should be recognized that P and Q could both be -O-C(O)NH(CH₂)₄- or -O-C(O)NH- or some other linkage fragment, as discussed below. The moiety represented by R is hydrogen, H. 20 The moiety represented by Z is the carboxyl group, -COOH. The moieties P, R, Q, and Z are all joined to a central carbon atom.

The nonpeptidic polymer arms, poly_a and poly_b, are mPEG moieties mPEG_a and mPEG_b, respectively, and are 25 the same on each of the linker fragments Q and P for the examples above. The mPEG moieties have a structure represented as CH₃O-(CH₂CH₂O)_nCH₂CH₂- . For the mPEG disubstituted lysine made by the one step method, n is about 454 to provide a molecular weight for each mPEG 30 moiety of 20,000 and a dimer molecular weight of 40,000. For the mPEG disubstituted lysine made by the two step method, n is about 114 to provide a molecular

-30-

weight for each mPEG moiety of 5,000 and a dimer molecular weight of 10,000.

Lysine disubstituted with mPEG and having as dimer molecular weights of 10,000 and 40,000 and 5 procedures for preparation of mPEG-disubstituted lysine have been shown. However, it should be recognized that mPEG disubstituted lysine and other multi-armed compounds of the invention can be made in a variety of molecular weights, including ultra high molecular 10 weights. High molecular weight monofunctional PEGs are otherwise difficult to obtain.

Polymerization of ethylene oxide to yield mPEGs usually produces molecular weights of up to about 20,000 to 25,000 g/mol. Accordingly, two-armed mPEG 15 disubstituted lysines of molecular weight of about 40,000 to 50,000 can be made according to the invention. Higher molecular weight lysine disubstituted PEGs can be made if the chain length of the linear mPEGs is increased, up to about 100,000. 20 Higher molecular weights can also be obtained by adding additional monofunctional nonpeptidic polymer arms to additional reactive sites on a linker moiety, within practical limits of steric hindrance. However, no unreacted active sites on the linker should remain that 25 could interfere with the monofunctionality of the multi-armed derivative. Lower molecular weight disubstituted mPEGs can also be made, if desired, down to a molecular weight of about 100 to 200.

It should be recognized that a wide variety 30 of linker fragments P and Q are available, although not necessarily with equivalent results, depending on the precursor linker moiety and the functional moiety with which the activated mPEG or other nonpeptidic monofunctional polymer is substituted and from which 35 the linker fragments result. Typically, the linker fragments will contain the reaction products of portions of linker moieties that have reactive amino

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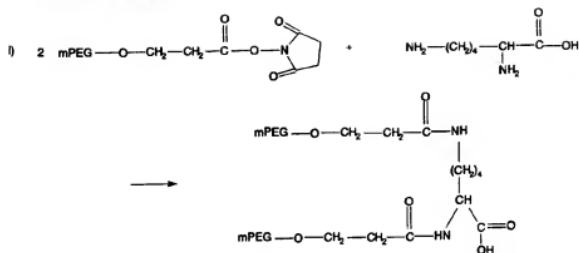
and/or thiol moieties and suitably activated nonpeptidic, monofunctional, water soluble polymers.

For example, a wide variety of activated mPEGs are available that form a wide variety of 5 hydrolytically stable linkages with reactive amino moieties. Linkages can be selected from the group consisting of amide, amine, ether, carbamate, which are also called urethane linkages, urea, thiourea, thiocarbamate, thiocarbonate, thioether, thioester, 10 dithiocarbonate linkages, and others. However, hydrolytically weak ester linkages and potentially toxic aromatic moieties are to be avoided.

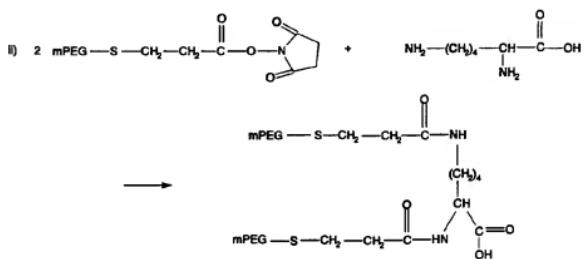
Hydrolytic stability of the linkages means that the linkages between the polymer arms and the 15 linker moiety are stable in water and that the linkages do not react with water at useful pHs for an extended period of time of at least several days, and potentially indefinitely. Most proteins could be expected to lose their activity at a caustic pH of 11 20 or higher, so the derivatives should be stable at a pH of less than about 11.

Examples of the above linkages and their formation from activated mPEG and lysine are represented structurally below.

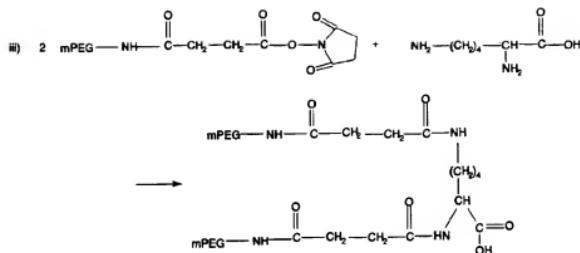
a) Formation of Amide Linkage



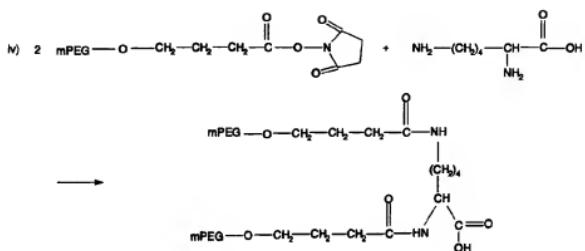
- 33 -



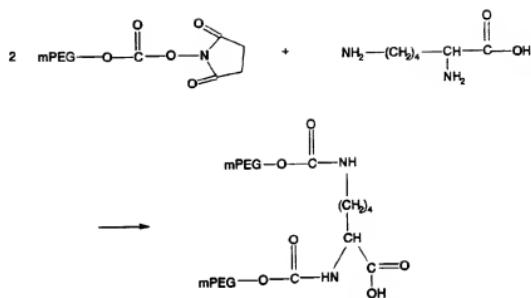
- 34 -



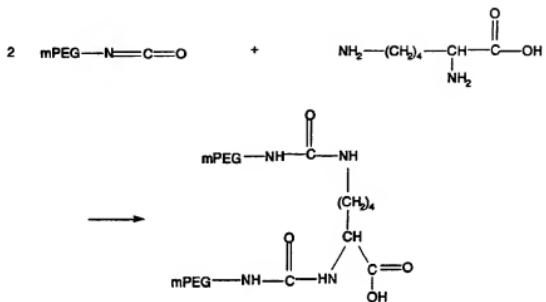
- 35 -



b) Formation of Carbamate Linkage

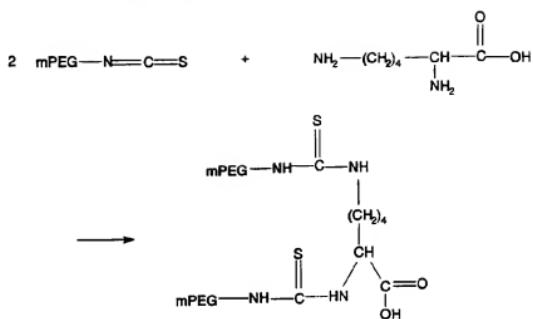


c) Formation of Urea Linkage

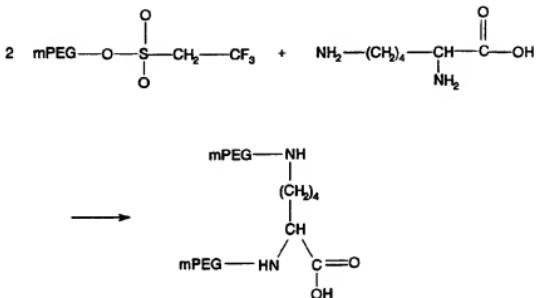


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d) Formation of Thiourea Linkage



e) Formation of Amine Linkage

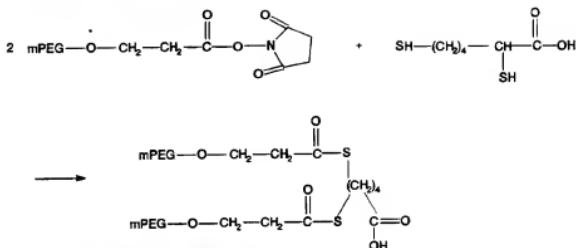


One or both of the reactive amino moieties, -NH₂, of lysine or another linker moiety can be replaced with thiol moieties, -SH. Where the linker moiety has a reactive thiol moiety instead of an amino moiety,

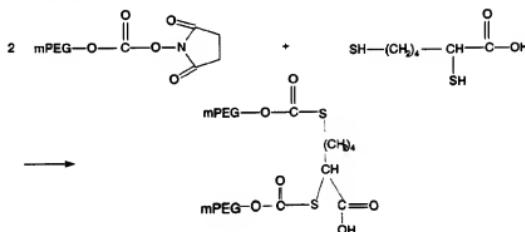
5 then the linkages can be selected from the group consisting of thioester, thiocarbonate, thiocarbamate, dithiocarbamate, thioether linkages, and others. The above linkages and their formation from activated mPEG and lysine in which both amino moieties have been

10 replaced with thiol moieties are represented structurally below.

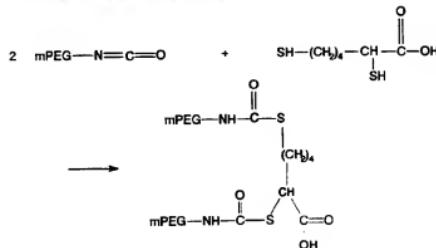
a) Formation of Thioester Linkage



b) Formation of Thiocarbonate Linkage

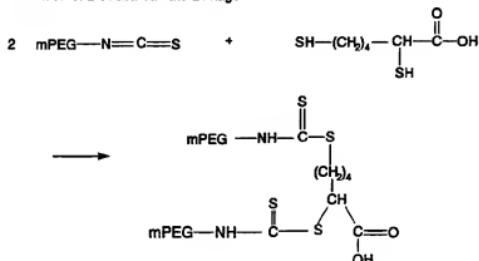


c) Formation of Thiocarbamate Linkage

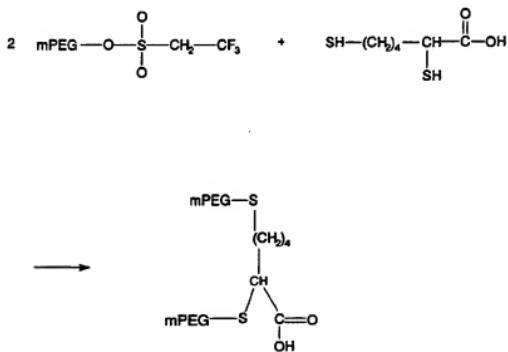


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d) Formation of Dithiocarbamate Linkage

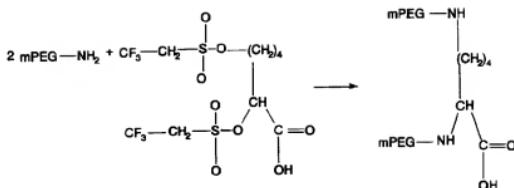


e) Formation of Thioether Linkage



It should be apparent that the mPEG or other monofunctional polymer reactants can be prepared with a reactive amino moiety and then linked to a suitable linker moiety having reactive groups such as those

5 shown above on the mPEG molecule to form hydrolytically stable linkages as discussed above. For example, the amine linkage could be formed as follows:



Examples of various active electrophilic moieties useful for activating polymers or linking moieties for biological and biotechnical applications in which the active moiety is reacted to form

- 5 hydrolytically stable linkages in the absence of aromatic moieties include trifluoroethylsulfonate, isocyanate, isostiocyanate, active esters, active carbonates, various aldehydes, various sulfones, including chloroethylsulfone and vinylsulfone,
- 10 maleimide, iodoacetamide, and iminoesters. Active esters include N-hydroxylsuccinimidyl ester. Active carbonates include N-hydroxylsuccinimidyl carbonate, p-nitrophenylcarbonate, and trichlorophenylcarbonate. These electrophilic moieties are examples of those that
- 15 are suitable as Ws in the structure poly-W and as Xs and Ys in the linker structure X-CR(-Y)-Z.

Nucleophilic moieties for forming the linkages can be amino, thiol, and hydroxyl. Hydroxyl moieties form hydrolytically stable linkages with

- 20 isocyanate electrophilic moieties. Also, it should be recognized that the linker can be substituted with different nucleophilic or electrophilic moieties or both electrophilic and nucleophilic moieties depending on the active moieties on the monofunctional polymers
- 25 with which the linker moiety is to be substituted.

Linker moieties other than lysine are available for activation and for disubstitution or

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multisubstitution with mPEG and related polymers for creating multi-armed structures in the absence of aromatic moieties in the structure and that are hydrolytically stable. Examples of such linker

5 moieties include those having more than one reactive site for attachment of various monofunctional polymers.

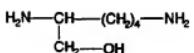
Linker moieties can be synthesized to include multiple reactive sites such as amino, thiol, or hydroxyl groups for joining multiple suitably activated

10 mPEGS or other nonpeptidic polymers to the molecule by hydrolytically stable linkages, if it is desired to design a molecule having multiple nonpeptidic polymer branches extending from one or more of the linker arm fragments. The linker moieties should also include a

15 reactive site, such as a carboxyl or alcohol moiety, represented as -Z in the general structure above, for which the activated polymers are not selective and that can be subsequently activated for selective reactions for joining to enzymes, other proteins, surfaces, and

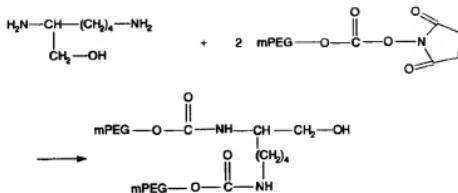
20 the like.

For example, one suitable linker moiety is a diamino alcohol having the structure



The diamino alcohol can be disubstituted with activated mPEG or other suitable activated polymers

25 similar to disubstitution of lysine and then the hydroxyl moiety can be activated as follows:



Other diamino alcohols and alcohols having more than two amino or other reactive groups for polymer attachment are useful. A suitably activated mPEG or other monofunctional, nonpeptidic, water soluble polymer can be attached to the amino groups on such a diamino alcohol similar to the method by which the same polymers are attached to lysine as shown above. Similarly, the amino groups can be replaced with thiol or other active groups as discussed above.

5 However, only one hydroxyl group, which is relatively nonreactive, should be present in the -Z moiety, and can be activated subsequent to polymer substitution.

10 The moiety -Z can include a reactive moiety or functional group, which normally is a carboxyl moiety, hydroxyl moiety, or activated carboxyl or hydroxyl moiety. The carboxyl and hydroxyl moieties are somewhat nonreactive as compared to the thiol, amino, and other moieties discussed above. The carboxyl and hydroxyl moieties typically remain intact 15 when the polymer arms are attached to the linker moiety and can be subsequently activated. The carboxyl and hydroxyl moieties also provide a mechanism for purification of the multisubstituted linker moiety. The carboxyl and hydroxyl moieties provide a site for 20 interacting with ion exchange chromatography media.

25 The moiety -Z may also include a linkage fragment, represented as R, in the moiety, which can be

substituted or unsubstituted, branched or linear, and joins the reactive moiety to the central carbon. Where a reactive group of the -Z moiety is carboxyl, for activation after substitution with nonpeptidic

5 polymers, then the -Z moiety has the structure -R_z-COOH if the R_z fragment is present. For hydroxyl, the structure is -R_z-OH. For example, in the diamino alcohol structure discussed above, R_z is CH₂. It should be understood that the carboxyl and hydroxyl moieties 10 normally will extend from the R_z terminus, but need not necessarily do so.

R_z can also include the reaction product of one or more reactive moieties including reactive amino, thiol, or other moieties, and a suitably activated MPEG 15 arm or related nonpeptidic polymer arm. In the latter event, R_z can have the structure (-L-poly_c)-COOH or (-L-poly_c)-OH in which -L- is the reaction product of a portion of the linker moiety and a suitably activated nonpeptidic polymer, poly_c-W, which is selected from the 20 same group as poly_a-W and poly_b-W but can be the same or different from poly_a-W and poly_b-W.

It is intended that -Z have a broad definition. The moiety -Z is intended to represent not only the reactive site of the multisubstituted 25 polymeric derivative that subsequently can be converted to an active form and its attachment to the central carbon, but the activated reactive site and also the conjugation of the precursor activated site with another molecule, whether that molecule be an enzyme, 30 other protein or polypeptide, a phospholipid, a preformed liposome, or on a surface to which the polymer derivative is attached.

The skilled artisan should recognize that Z encompasses the currently known activating moieties in 35 PEG chemistry and their conjugates. It should also be recognized that, although the linker fragments represented by Q and P and R_z should not contain

aromatic rings or hydrolytically weak linkages such as ester linkages, such rings and such hydrolytically weak linkages may be present in the active site moiety of -Z or in a molecule joined to such active site. It may 5 be desirable in some instances to provide a linkage between, for example, a protein or enzyme and a multisubstituted polymer derivative that has limited stability in water. Some amino acids contain aromatic moieties, and it is intended that the structure Z 10 include conjugates of multisubstituted monofunctional polymer derivatives with such molecules or portions of molecules. Activated Zs and Zs including attached proteins and other moieties are discussed below.

When lysine, the diamino alcohol shown above, 15 or many other compounds are linkers, then the central carbon has a nonreactive hydrogen, H, attached thereto. In the general structure poly_a-P-CR(-Q-poly_b)-Z, R is H. It should be recognized that the moiety R can be designed to have another substantially nonreactive 20 moiety, such as a nonreactive methyl or other alkyl group, or can be the reaction product of one or more reactive moieties including reactive amino, thiol, or other moieties, and a suitably activated mPEG arm or related nonpeptidic polymer arm. In the latter event, 25 R can have the structure -M-poly_a, in which -M- is the reaction product of a portion of the linker moiety and a suitably activated nonpeptidic polymer, poly_a-W, which is selected from the same group as poly_a-W and poly_b-W but can be the same or different from poly_a-W 30 and poly_b-W.

For example, multi-armed structures can be made having one or more mPEGS or other nonpeptidic polymer arms extending from each portion P, Q, R, and R_z, all of which portions extend from a central carbon 35 atom, C, which multi-armed structures have a single reactive site for subsequent activation included in the structure represented by Z. Upon at least the linker

fragments P and Q are located at least one active site for which the monofunctional, nonpeptidic polymers are selective. These active sites include amino moieties, thiol moieties, and other moieties as described above.

5 The nonpeptidic polymer arms tend to mask antigenic properties of the linker fragment, if any. A linker fragment length of from 1 to 10 carbon atoms or the equivalent has been determined to be useful to avoid a length that could provide an antigenic site.

10 Also, for all the linker fragments P, Q, R, and R₂, there should be an absence of aromatic moieties in the structure and the linkages should be hydrolytically stable.

 Poly(ethylene glycol) is useful in the practice of the invention for the nonpeptidic polymer arms attached to the linker fragments. PEG is used in biological applications because it has properties that are highly desirable and is generally approved for biological or biotechnical applications. PEG typically is clear, colorless, odorless, soluble in water, stable to heat, inert to many chemical agents, does not hydrolyze or deteriorate, and is nontoxic.

 Poly(ethylene glycol) is considered to be biocompatible, which is to say that PEG is capable of coexistence with living tissues or organisms without causing harm. More specifically, PEG is not immunogenic, which is to say that PEG does not tend to produce an immune response in the body. When attached to a moiety having some desirable function in the body, the PEG tends to mask the moiety and can reduce or eliminate any immune response so that an organism can tolerate the presence of the moiety. Accordingly, the activated PEGs of the invention should be substantially non-toxic and should not tend substantially to produce an immune response or cause clotting or other undesirable effects.

The term "PEG" is used in the art and herein to describe any of several condensation polymers of ethylene glycol having the general formula represented by the structure



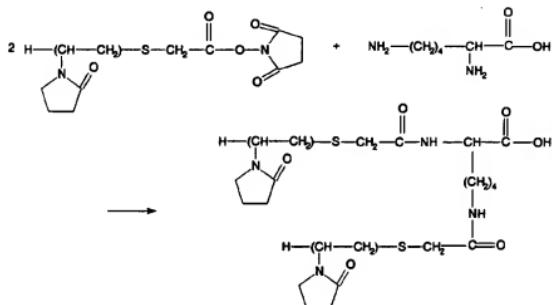
5 or, more simply, as HO-PEG-OH. PEG is also known as polyoxyethylene, polyethylene oxide, polyglycol, and polyether glycol. PEG can be prepared as copolymers of ethylene oxide and many other monomers.

10 Other water soluble polymers than PEG are suitable for similar modification to create multi-armed structures that can be activated for selective reactions. These other polymers include poly(vinyl alcohol) ("PVA"); other poly(alkylene oxides) such as poly(propylene glycol) ("PPG") and the like; and 15 poly(oxyethylated polyols) such as poly(oxyethylated glycerol), poly(oxyethylated sorbitol), and poly(oxyethylated glucose), and the like. The polymers can be homopolymers or random or block copolymers and terpolymers based on the monomers of the above 20 polymers, straight chain or branched, or substituted or unsubstituted similar to mPEG and other capped, monofunctional PEGs having a single active site available for attachment to a linker.

Specific examples of suitable additional 25 polymers include poly(oxazoline), poly(acryloylmorpholine) ("PAcM"), and poly(vinylpyrrolidone) ("PVP"). PVP and poly(oxazoline) are well known polymers in the art and their preparation and use in the syntheses described above 30 for mPEG should be readily apparent to the skilled artisan.

An example of the synthesis of a PVP disubstituted lysine having a single carboxyl moiety

available for activation is shown below. The disubstituted compound can be purified, activated, and used in various reactions for modification of molecules and surfaces similarly to the mPEG-disubstituted lysine 5 described above.

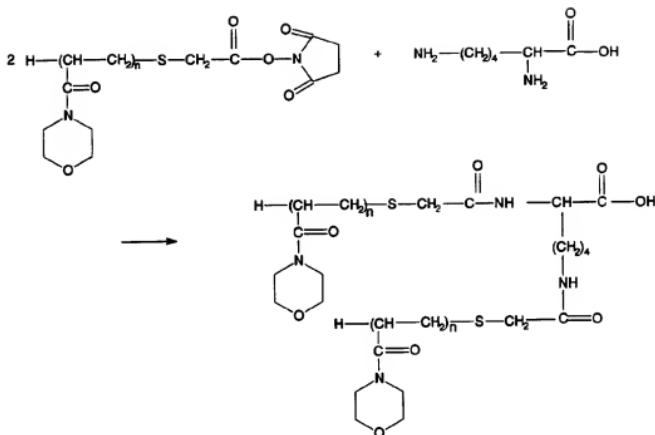


Poly(acryloylmorpholine) "(PAcM)" functionalized at one end is a new polymer, the structure, preparation, and characteristics of which are described in Italian Patent Application No. MI 92 A 10 0002616, which was published May 17, 1994 and is entitled, in English, "*Polymers Of N-Acryloylmorpholine Functionalized At One End And Conjugates With Bioactive Materials And Surfaces.*" Dimer polymers of molecular weight up to at least about 80,000 can be prepared 15 using this polymer. The contents of the Italian patent application are incorporated herein by reference.

PAcM can be used similarly to mPEG or PVP to create multi-armed structures and ultra-high molecular weight polymers. An example of a PAcM-disubstituted 20 lysine having a single carboxyl moiety available for activation is shown below. The disubstituted compound can be purified, activated, and used in various reactions for modification of molecules and surfaces

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similarly to the mPEG- and PVP-disubstituted lysines described above.



It should also be recognized that the multi-armed monofunctional polymers of the invention can be used for attachment to a linker moiety to create a highly branched monofunctional structure, within the practical limits of steric hindrance.

II. Activation of mPEG-Disubstituted Lysine and Modification of Protein Amino Groups.

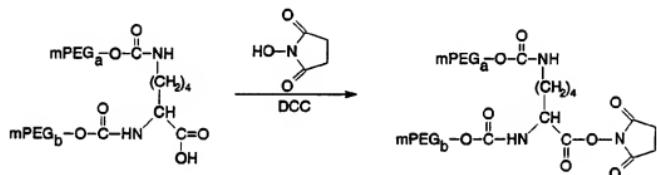
Schemes are represented below for activating the mPEG-disubstituted lysine product made by either the one step or two step procedures and for linking the activated mPEG-disubstituted lysine through a stable carbamate linkage to protein amino groups to prepare

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polymer and protein conjugates. Various other multisubstituted polymer derivatives as discussed above can be activated similarly.

A. Activation of mPEG Disubstituted Lysine.

5 Purified mPEG-disubstituted lysine produced in accordance with the two step procedure discussed above was activated with N-hydroxysuccinimide to produce mPEG-disubstituted lysine activated as the succinimidyl ester. The reaction is represented
10 structurally below:



Six and two tenths grams of mPEG-disubstituted lysine of molecular weight 10,000, which is about 0.6 millimoles, was dissolved in 10 milliliters of anhydrous methylene chloride and cooled 15 to 0°C. N-hydroxysuccinimide and N,N-dicyclohexylcarbodiimide ("DCC") were added under stirring in the amounts, respectively, of 0.138 milligrams, which is about 1.2 millimoles, and 0.48 milligrams, which is about 1.2 millimoles. The 20 reaction mixture was stirred overnight at room temperature. Precipitated dicyclohexylurea was removed by filtration and the solution was concentrated and precipitated with diethyl ether. The product, mPEG disubstituted lysine activated as the succinimidyl ester, was crystallized from ethyl acetate. The yield of esterification, calculated on the basis of hydroxysuccinimide absorption at 260 nm (produced by hydrolysis), was over 97% (ϵ of hydroxysuccinimide at 260 nm = $8,000 \text{ m}^{-1}\text{cm}^{-1}$). The NMR spectrum was identical
25

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to that of the unactivated carboxylic acid except for the new succinimide singlet at 2.80 ppm (2Hs)

The procedure previously described for the activation of the mPEG-disubstituted lysine of 5 molecular weight 10,000 was also followed for the activation of the higher molecular weight polymer of molecular weight approximately 40,000 that was produced in accordance with the one step procedure discussed above. The yield was over 95% of high molecular weight 10 mPEG-disubstituted lysine activated as the succinimidyl ester.

It should be recognized that a number of activating groups can be used to activate the multisubstituted polymer derivatives for attachment to 15 surfaces and molecules. Any of the activating groups of the known derivatives of PEG can be applied to the multisubstituted structure. For example, the mPEG-disubstituted lysine of the invention was functionalized by activation as the succinimidyl ester, 20 which can be attached to protein amino groups. However, there are a wide variety of functional moieties available for activation of carboxilic acid polymer moieties for attachment to various surfaces and molecules. Examples of active moieties used for 25 biological and biotechnical applications include trifluoroethylsulfonate, isocyanate, isothiocyanate, active esters, active carbonates, various aldehydes, various sulfones, including chloroethylsulfone and vinylsulfone, maleimide, iodoacetamide, and 30 iminoesters. Active esters include N-hydroxylsuccinimidyl ester. Active carbonates include N-hydroxylsuccinimidyl carbonate, p-nitrophenylcarbonate, and trichlorophenylcarbonate.

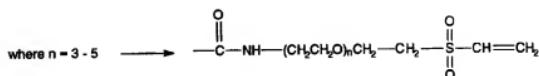
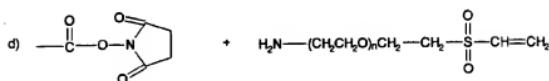
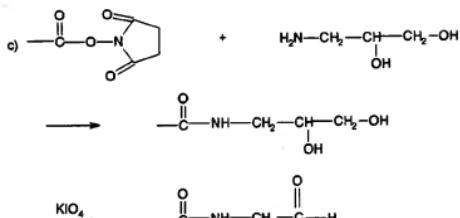
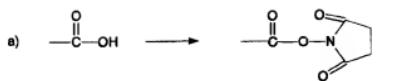
A highly useful, new activating group that 35 can be used for highly selective coupling with thiol moieties instead of amino moieties on molecules and surfaces is the vinyl sulfone moiety described in co-

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pending U.S. Patent Application No. 08/151,481, which was filed on November 12, 1993, the contents of which are incorporated herein by reference. Various sulfone moieties can be used to activate a multi-armed

5 structure in accordance with the invention for thiol selective coupling.

Various examples of activation of -Z reactive moieties to created -Z activated moieties are presented as follows:

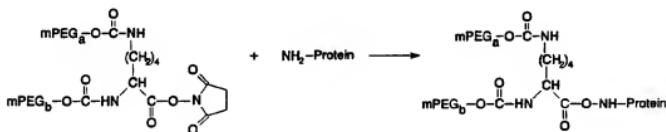


It should also be recognized that, although the linker fragments represented by Q and P should not contain aromatic rings or hydrolytically weak linkages such as ester linkages, such rings and such

5 hydrolytically weak linkages may be present in the moiety represented by -Z. It may be desirable in some instances to provide a linkage between, for example, a protein or enzyme and a multisubstituted polymer derivative that has limited stability in water. Some 10 amino acids contain aromatic moieties, and it is intended that the structure -Z include conjugates of multisubstituted monofunctional polymer derivatives with such molecules or portions of molecules.

B. Enzyme Modification

15 Enzymes were modified with activated, two-armed, mPEG-disubstituted lysine of the invention of molecular weight about 10,000 that had been prepared according to the two step procedure and activated as the succinimidyl ester as discussed above. The 20 reaction is represented structurally below:



For comparison, enzymes were also modified with activated, conventional, linear mPEG of molecular weight 5,000, which was mPEG with a norleucine amino acid spacer arm activated as the succinimide. In the 25 discussion of enzyme modification below, conventional, linear mPEG derivatives with which enzymes are modified are referred to as "linear mPEG." The activated, two-armed, mPEG-disubstituted lysine of the invention is referred to as "two-armed mPEG." Different procedures 30 were used for enzyme modification depending upon the

type of enzyme and the polymer used so that a similar extent of amino group modification or attachment for each enzyme could be obtained. Generally, higher molar ratios of the two-armed mPEG were used. However, in 5 all examples the enzymes were dissolved in a 0.2 M borate buffer of pH 8.5 to dissolve proteins. The polymers were added in small portions for about 10 minutes and stirred for over 1 hour. The amount of polymer used for modification was calculated on the 10 basis of available amino groups in the enzyme.

Ribonuclease in a concentration of 1.5 milligrams per milliliter of buffer was modified at room temperature. Linear and two-armed mPEGs as described were added at a molar ratio of polymer to 15 protein amino groups of 2.5:1 and 5:1, respectively. Ribonuclease has a molecular weight of 13,700 D and 11 available amino groups. Catalase has a molecular weight of 250,000 D with 112 available amino groups. Trypsin has a molecular weight of 23,000 D with 16 20 available amino groups. Erwinia Caratimora asparaginase has a molecular weight of 141,000 D and 92 free amino groups.

Catalase in a concentration of 2.5 milligrams per milliliter of buffer was modified at room 25 temperature. Linear and two-armed mPEGs as described were added at a molar ratio of polymer to protein amino groups of 5:1 and 10:1, respectively.

Trypsin in a concentration of 4 milligrams per milliliter of buffer was modified at 0°C. Linear 30 and two-armed mPEGs as described were added at a molar ratio of polymer to protein amino groups of 2.5:1.

Asparaginase in a concentration of 6 milligrams per milliliter of buffer was modified with linear mPEG at room temperature. Linear mPEG as 35 described was added at a molar ratio of polymer to protein amino groups of 3:1. Asparaginase in a concentration of 6 milligrams per milliliter of buffer

was modified with two-armed mPEG at 37°C. Two-armed mPEG of the invention as described was added at a molar ratio of polymer to protein amino groups of 3.3:1.

The polymer and enzyme conjugates were

5 purified by ultrafiltration and concentrated in an Amicon system with a PM 10 membrane (cut off 10,000) to eliminate N-hydroxysuccinimide and reduce polymer concentration. The conjugates were further purified from the excess of unreacted polymer by gel filtration

10 chromatography on a Pharmacia Superose 12 column, operated by an FPLC instrument, using 10 mM phosphate buffer of pH 7.2, 0.15 M in NaCl, as eluent.

Protein concentration for the native forms of ribonuclease, catalase, and trypsin was evaluated

15 spectrophotometrically using molar extinction coefficients of $945 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $1.67 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and $3.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm, respectively. The concentration of native asparaginase was evaluated by biuret assay. Biuret assay was also used to evaluate

20 concentrations of the protein modified forms.

The extent of protein modification was evaluated by one of three methods. The first is a colorimetric method described in Habeeb, A. F. S. A. (1966) Determination of free amino groups in protein by

25 trinitrobenzensulphonic acid. *Anal. Biochem.* 14, 328-336. The second method is amino acid analysis after acid hydrolysis. This method was accomplished by two procedures: 1) the post-column procedure of Benson, J. V., Gordon, M. J., and Patterson, J. A. (1967)

30 Accelerated chromatographic analysis of amino acid in physiological fluids containing vitamin and asparagine. *Anal. Biol. Chem.* 18, 288-333, and 2) pre-column derivatization by phenylisothiocyanate (PITC) according to Bidlingmeyer, B. A., Cohen, S. A., and Tarvin, T. L. (1984) Rapid analysis of amino acids using pre-column derivatization. *J. Chromatography* 336, 93-104.

The amount of bound linear mPEG was evaluated from norleucine content with respect to other protein amino acids. The amount of two-armed, mPEG-disubstituted lysine was determined from the increase 5 in lysine content. One additional lysine is present in the hydrolysate for each bound polymer.

III. Analysis of Polymer and Enzyme Conjugates

Five different model enzymes, ribonuclease, catalase, asparaginase, trypsin and uricase, were 10 modified with linear, conventional mPEG of molecular weight 5000 having a norleucine amino acid spacer arm activated as succinimidyl ester and with a two-armed, mPEG-disubstituted lysine of the invention prepared from the same linear, conventional mPEG as described 15 above in connection with the two step procedure. The molecular weight of the two-armed mPEG disubstituted lysine of the invention was approximately 10,000.

A. Comparison of Enzyme Activity. The catalytic properties of the modified enzymes were 20 determined and compared and the results are presented in Table 1 below. To facilitate comparison, each enzyme was modified with the two polymers to a similar extent by a careful choice of polymer to enzyme ratios and reaction temperature.

Ribonuclease with 50% and 55% of the amino 25 groups modified with linear mPEG and two-armed mPEG, respectively, presented 86% and 94% residual activity with respect to the native enzyme. Catalase was modified with linear mPEG and with two-armed mPEG to obtain 43% and 38% modification of protein amino 30 groups, respectively. Enzyme activity was not significantly changed after modification. Trypsin modification was at the level of 50% and 57% of amino groups with linear mPEG and with two-armed mPEG, respectively. Esterolytic activity for enzyme modified 35 with linear mPEG and two-armed mPEG, assayed on the small substrate TAME, was increased by the modification

to 120% and 125%, respectively. Asparaginase with 53% and 40% modified protein amino groups was obtained by coupling with linear mPEG and two-armed mPEG, respectively. Enzymatic activity was increased, 5 relative to the free enzyme, to 110% for the linear mPEG conjugate and to 133% for the two-armed mPEG conjugate.

While not wishing to be bound by theory, it is possible that in the case of trypsin and 10 asparaginase, that modification produces a more active form of the enzyme. The K_m values of the modified and unmodified forms are similar.

For the enzyme uricase a particularly dramatic result was obtained. Modification of uricase 15 with linear mPEG resulted in total loss of activity. While not wishing to be bound by theory, it is believed that the linear mPEG attached to an amino acid such as lysine that is critical for activity. In direct contrast, modification of 40% of the lysines of uricase 20 with two-armed mPEG gave a conjugate retaining 70% activity.

It is apparent that modification of enzymes with two-armed mPEG gives conjugates of equal or greater activity than those produced by conventional 25 linear mPEG modification with monosubstituted structures, despite the fact that two-armed mPEG modification attaches twice as much polymer to the enzyme.

Coupling two-armed mPEG to asparaginase with 30 chlorotriazine activation as described in the background of the invention gave major loss of activity. Presumably the greater activity of enzymes modified with a two-armed mPEG of the invention results because the bulky two-armed mPEG structure is less 35 likely than monosubstituted linear mPEG structures to penetrate into active sites of the proteins.

Table 1. Properties of enzymes modified by linear mPEG and two-armed mPEG.

ENZYME ^a	NH ₂ :POLYMER MOLAR RATIO	% MODIFICATION	% ACTIVITY	Km (M)	Kcas (min ⁻¹)
5 Ribonuclease RN RP1 RP2	1:0	0	100		
	1:2.5	50	86		
	1:5	55	94		
10 Catalase CN CP1 CP2	1:0	0	100		
	1:5	43	100		
	1:10	38	90		
15 Trypsin ^b TN TP1 TP2	1:0	0	100	8.2x10 ⁻⁵	830
	1:2.5	50	120	7.6x10 ⁻⁵	1790
	1:2.5	57	125	8.0x10 ⁻⁵	2310
20 Asparaginase AN AP1 AP2	1:0	0	100	3.31x10 ⁻⁶	523
	1:3	53	110	3.33x10 ⁻⁶	710
	1:3.3	40	133	3.30x10 ⁻⁶	780
Uricase UP UP1 UP2	1:0	0	100		
	1:5	45	0		
	1:10	40	70		

^aN = native enzyme, P1 = enzyme modified with linear mPEG, P2 = enzyme modified with two-armed mPEG.

^b For trypsin only the esterolytic activity is reported.

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Enzymatic activity of native and modified enzyme was evaluated by the following methods. For ribonuclease, the method was used of Crook, E. M., Mathias, A. P., and Rabin, B.R. (1960)

5 Spectrophotometric assay of bovine pancreatic ribonuclease by the use of cytidine 2':3' phosphate. Biochem. J. 74, 234-238. Catalase activity was determined by the method of Beers, R. F. and Sizer, I. W. (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133-140. The esterolytic activity of trypsin and its derivatives was determined by the method of Laskowski, M. (1955) Trypsinogen and trypsin. Methods Enzymol. 2, 26-36. Native and modified asparaginase 15 were assayed according to a method reported by Cooney, D. A., Capizzi, R. L. and Handschumacher, R. E. (1970) Evaluation of L-asparagine metabolism in animals and man. Cancer Res. 30, 929-935. In this method, 1.1 ml containing 120 µg of α-ketoglutaric acid, 20 U/l of 20 glutamic-oxalacetic transaminase, 30 U/l of malate dehydrogenase, 100 µg of NADH, 0.5 µg of asparaginase and 10 µmoles of asparagine were incubated in 0.122 M Tris buffer, pH 8.35, while the NADH absorbance decrease at 340 nm was followed.

25 B. Proteolytic Digestion of Free Enzyme and Conjugates. The rates at which proteolytic enzymes digest and destroy proteins was determined and compared for free enzyme, enzyme modified by attachment of linear activated mPEG, and enzyme modified by 30 attachment of an activated two-armed mPEG of the invention. The proteolytic activities of the conjugates were assayed according to the method of Zwilling, R., and Neurath, H. (1981) Invertrebate protease. Methods Enzymol. 80, 633-664. Four enzymes 35 were used: ribonuclease, catalase, trypsin, and asparaginase. From each enzyme solution, aliquots were

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taken at various time intervals and enzyme activity was assayed spectrophotometrically.

Proteolytic digestion was performed in 0.05 M phosphate buffer of pH 7.0. The free enzyme, linear 5 mPEG and protein conjugate, and two-armed mPEG-protein conjugates were exposed to the known proteolytic enzymes trypsin, pronase, elastase or subtilisin under conditions as follows.

For native ribonuclease and its linear and 10 two-armed mPEG conjugates, 0.57 mg protein was digested at room temperature with 2.85 mg of pronase, or 5.7 mg of elastase, or with 0.57 mg of subtilisin in a total volume of 1 ml. Ribonuclease with 50% and 55% of the amino groups modified with linear mPEG and two-armed 15 mPEG, respectively, was studied for stability to proteolytic digestion by pronase (Figure 1(a)), elastase (Figure 1(b)) and subtilisin (Figure 1(c)). Polymer modification greatly increases the stability to digestion by all three proteolytic enzymes, but the 20 protection offered by two-armed mPEG is much more effective as compared to linear mPEG.

For native and linear and two-armed mPEG-modified catalase, 0.58 mg of protein were digested at room temperature with 0.58 mg of trypsin or 25 3.48 mg of pronase in a total volume of 1 ml. Catalase was modified with linear mPEG and two-armed mPEG to obtain 43% and 38% modification of protein amino groups, respectively. Proteolytic stability was much greater for the two-armed mPEG derivative than for the 30 monosubstituted mPEG derivative, particularly toward pronase (Figure 3(a)) and trypsin (Figure 3(b)), where no digestion took place.

Autolysis of trypsin and its linear and two-armed mPEG derivatives at 37°C was evaluated by 35 esterolytic activity of protein solutions at 25 mg/ml of TAME. Trypsin modification was at the level of 50% and 57% of amino groups with linear mPEG and two-armed

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mPEG, respectively. Modification with linear mPEG and two-armed mPEG reduced proteolytic activity of trypsin towards casein, a high molecular weight substrate:

activity relative to the native enzyme was found, after

5 20 minutes incubation, to be 64% for the linear mPEG and protein conjugate and only 35% for the two-armed mPEG conjugate. In agreement with these results, the trypsin autolysis rate (i.e., the rate at which trypsin digests trypsin), evaluated by enzyme esterolytic

10 activity, was totally prevented in two-armed mPEG-trypsin but only reduced in the linear mPEG-trypsin conjugate. To prevent autolysis with linear mPEG, modification of 78% of the available protein amino groups was required.

15 For native and linear mPEG- and two-armed mPEG-modified asparaginase, 2.5 μ g were digested at 37°C with 0.75 mg of trypsin in a total volume of 1 ml. Asparaginase with 53% and 40% modified protein amino groups was obtained by coupling with linear mPEG and 20 two-armed mPEG, respectively. Modification with two-armed mPEG had an impressive influence on stability towards proteolytic enzyme. Increased protection was achieved at a lower extent of modification with respect to the derivative obtained with the two-armed polymer 25 (Figure 5).

These data clearly show that two-armed mPEG coupling is much more effective than conventional linear mPEG coupling in providing a protein with protection against proteolysis. While not wishing to 30 be bound by theory, it is believed that the two-armed mPEG, having two polymer chains bound to the same site, presents increased hindrance to approaching macromolecules in comparison to linear mPEG.

C. Reduction of Protein Antigenicity.

35 Protein can provoke an immune response when injected into the bloodstream. Reduction of protein immunogenicity by modification with linear and

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two-armed mPEG was determined and compared for the enzyme superoxidizedismutase ("SOD").

Anti-SOD antibodies were obtained from rabbit and purified by affinity chromatography. The antigens 5 (SOD, linear mPEG-SOD, and two-armed mPEG-SOD) were labelled with tritiated succinimidyl propionate to facilitate tracing. Reaction of antigen and antibody were evaluated by radioactive counting. In a 500 μ L sample, the antigen (in the range of 0-3 μ g) was 10 incubated with 2.5 μ g of antibody. The results show the practical disappearance of antibody recognition for two-armed mPEG-SOD, while an appreciable antibody-antigen complex was formed for linear mPEG-SOD and native SOD.

15 D. Blood Clearance Times. Increased blood circulation half lives are of enormous pharmaceutical importance. The degree to which mPEG conjugation of proteins reduces kidney clearance of proteins from the 20 blood was determined and compared for free protein, protein modified by attachment of conventional, linear activated mPEG, and protein modified by attachment of the activated two-armed mPEG of the invention. Two proteins were used. These experiments were conducted by assaying blood of mice for the presence of the 25 protein.

For linear mPEG-uricase and two-armed mPEG-uricase, with 40% modification of lysine groups, the half life for blood clearance was 200 and 350 minutes, respectively. For unmodified uricase the 30 result was 50 minutes.

For asparaginase, with 53% modification with mPEG and 40% modification with two armed mPEG, the half lives for blood clearance were 1300 and 2600 minutes, respectively. For unmodified asparaginase the result 35 was 27 minutes.

E. Thermal Stability of Free and Conjugated Enzymes. Thermal stability of native ribonuclease,

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catalase and asparaginase and their linear mPEG and two-armed mPEG conjugates was evaluated in 0.5 M phosphate buffer pH 7.0 at 1 mg/ml, 9 μ g/ml and 0.2 mg/ml respectively. The samples were incubated at the 5 specified temperatures for 15 min., 10 min., and 15 min, respectively, cooled to room temperature and assayed spectrophotometrically for activity.

Increased thermostability was found for the modified forms of ribonuclease, as shown in Figure 2, 10 at pH 7.0, after 15 min. incubation at different temperatures, but no significant difference between the two polymers was observed. Data for catalase, not reported here, showed that modification did not influence catalase thermostability. A limited increase 15 in thermal stability of linear and two-armed mPEG-modified asparaginase was also noted, but is not reported.

F. pH Stability of the Free and Conjugated Enzymes. Unmodified and polymer-modified enzymes were 20 incubated for 20 hrs in the following buffers: sodium acetate 0.05 M at a pH of from 4.0 to 6.0, sodium phosphate 0.05 M at pH 7.0 and sodium borate 0.05 M at a pH of from 8.0 to 11. The enzyme concentrations were 1 mg/ml, 9 μ g/ml, 5 μ g/ml for ribonuclease, catalase, 25 and asparaginase respectively. The stability to incubation at various pH was evaluated on the basis of enzyme activity.

As shown in Figure 2b, a decrease in pH stability at acid and alkline pH values was found for 30 the linear and two-armed mPEG-modified ribonuclease forms as compared to the native enzyme. As shown in Figure 4, stability of the linear mPEG and two-armed mPEG conjugates with catalase was improved for incubation at low pH as compared to native catalase. 35 However, the two-armed mPEG and linear mPEG conjugates showed equivalent pH stability. A limited increase in pH stability at acid and alkaline pH values was noted

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for linear and two-armed mPEG-modified asparaginase as compared to the native enzyme.

It should be recognized that there are thousands of proteins and enzymes that can be usefully modified by attachment to the polymer derivatives of the invention. Proteins and enzymes can be derived from animal sources, humans, microorganisms, and plants and can be produced by genetic engineering or synthesis. Representatives include: cytokines such as 10 various interferons (e.g. interferon- α , interferon- β , interferon- γ), interleukin-2 and interleukin-3), hormones such as insulin, growth hormone-releasing factor (GRF), calcitonin, calcitonin gene related peptide (CGRP), atrial natriuretic peptide (ANP), 15 vasopressin, corticotropin-releasing factor (CRF), vasoactive intestinal peptide (VIP), secretin, α -melanocyte-stimulating hormone (α -MSH), adrenocorticotrophic hormone (ACTH), cholecystokinin (CCK), glucagon, parathyroid hormone (PTH), 20 somatostatin, endothelin, substance P, dynorphin, oxytocin and growth hormone-releasing peptide, tumor necrosis factor binding protein, growth factors such as growth hormone (GH), insulin-like growth factor (IGF-I, IGF-II), β -nerve growth factor (β -NGF), basic 25 fibroblast growth factor (bFGF), transforming growth factor, erythropoietin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), 30 enzymes such as tissue plasminogen activator (t-PA), elastase, superoxide dismutase (SOD), bilirubin oxydase, catalase, uricase and asparaginase, other proteins such as ubiquitin, islet activating protein (IAP), serum thymic factor (STF), peptide-T and trypsin 35 inhibitor, and derivatives thereof. In addition to protein modification, the two-armed polymer derivative of the invention has a variety of related applications.

Small molecules attached to two-armed activated mPEG derivatives of the invention can be expected to show enhanced solubility in either aqueous or organic solvents. Lipids and liposomes attached to the 5 derivative of the invention can be expected to show long blood circulation lifetimes. Other particles than lipids and surfaces having the derivative of the invention attached can be expected to show nonfouling characteristics and to be useful as biomaterials having 10 increased blood compatibility and avoidance of protein adsorption. Polymer-ligand conjugates can be prepared that are useful in two phase affinity partitioning. The polymers of the invention could be attached to various forms of drugs to produce prodrugs. Small 15 drugs having the multisubstituted derivative attached can be expected to show altered solubility, clearance time, targeting, and other properties.

The invention claimed herein has been described with respect to particular exemplified 20 embodiments. However, the foregoing description is not intended to limit the invention to the exemplified embodiments, and the skilled artisan should recognize that variations can be made within the scope and spirit of the invention as described in the foregoing 25 specification. The invention includes all alternatives, modifications, and equivalents that may be included within the true spirit and scope of the invention as defined by the appended claims.

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WHAT IS CLAIMED IS:

1. A polymeric derivative represented by the structure



wherein poly_a and poly_b are nonpeptidic and substantially nonreactive water soluble polymeric arms that may be the 5 same or different, wherein C is carbon, wherein P and Q comprise linkage fragments that may be the same or different and join polymeric arms poly_a and poly_b, respectively, to C by hydrolytically stable linkages in the absence of aromatic rings in said linkage fragments, 10 wherein R is a moiety selected from the group consisting of H, substantially nonreactive moieties, and linkage fragments having attached thereto by a hydrolytically stable linkage in the absence of aromatic rings one or more nonpeptidic and substantially nonreactive water 15 soluble polymeric arms, and wherein Z comprises a moiety selected from the group consisting of moieties having a single site reactive toward nucleophilic moieties, sites that can be converted to sites reactive toward nucleophilic moieties, and the reaction product of a 20 nucleophilic moiety and moieties having a single site reactive toward nucleophilic moieties.

2. The polymeric derivative of Claim 1 wherein said hydrolytically stable linkages are selected from the group consisting of amide, amine, ether, 25 carbamate, thiourea, urea, thiocarbamate, thiocarbonate, thioether, thioester, and dithiocarbamate linkages.

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3. The polymeric derivative of Claim 1 wherein said nucleophilic moieties are selected from the group consisting of amino, thiol, and hydroxyl moieties.

5 4. The polymeric derivative of Claim 1 wherein said nucleophilic moiety is a biologically active molecule.

5 10 5. The polymeric derivative of Claim 4 wherein said biologically active molecule is selected from the group consisting of polypeptides, polynucleotides, and lipids.

6. The polymeric derivative of Claim 1 wherein said nucleophilic moiety is a solid surface or a particle.

15 7. The polymeric derivative of Claim 6 wherein said solid particle is a liposome.

8. The polymeric derivative of Claim 1 wherein Z is selected from the group consisting of 20 carboxyl, hydroxyl, activated carboxyl, activated hydroxyl, and conjugates of activated carboxyl or hydroxyl sites and molecules having at least one reactive nucleophilic moiety.

9. The polymeric derivative of Claim 1 25 wherein Z comprises a moiety selected from the group consisting of trifluoroethylsulfonate, isocyanate, isothiocyanate, active esters, active carbonates, aldehyde, vinylsulfone, maleimide, iodoacetamide, and iminoesters.

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10. The polymeric derivative of Claim 9
wherein said active ester is N-hydroxylsuccinimidyl
ester and said active carbonates are selected from the
group consisting of N-hydroxylsuccinimidyl carbonate,
5 *p*-nitrophenylcarbonate, and trichlorophenylcarbonate.

11. The polymeric derivative of Claim 1
wherein said nonpeptidic polymeric arms are selected
from the group consisting of poly(alkylene oxides),
poly(oxyethylated polyols), and poly(oxyethylated
10 glucose).

12. The polymeric derivative of Claim 1
wherein said nonpeptidic polymeric arms are selected
from the group consisting of poly(ethylene glycol),
poly(vinyl alcohol), poly(propylene glycol),
15 poly(oxyethylated glycerol), poly(oxyethylated
sorbitol), poly(oxyethylated glucose), poly(oxazoline),
poly(acryloylmorpholine), and poly(vinylpyrrolidone).

13. The polymeric derivative of Claim 1
wherein said nonpeptidic polymeric arms are linear
20 mPEGs of molecular weight of from about 50 to 50,000.

14. The polymeric derivative of Claim 1
wherein said linkage fragments P and Q comprise
hydrolytically stable linkages in the absence of
aromatic rings to one or more nonpeptidic and water
25 soluble polymeric arms.

15. The polymeric derivative of Claim 1
wherein R comprises a linkage fragment attached by a
hydrolytically stable linkage in the absence of
aromatic rings to a nonpeptidic and substantially
30 nonreactive water soluble polymeric arm.

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16. The polymeric derivative of Claim 15
wherein R is represented by the general structure
-M-poly_a, wherein poly_a is said polymeric arm and M is
said linkage fragment.

5 17. The polymeric derivative of Claim 1
wherein Z further comprises a linkage fragment attached
by a hydrolytically stable linkage in the absence of
aromatic rings to a nonpeptidic and substantially
nonreactive water soluble polymeric arm.

10 18. A polymeric derivative represented by
the structure



wherein poly_a and poly_b may be the same or different and
are selected from the group consisting of linear
poly(ethylene glycol), poly(vinyl alcohol),
15 poly(propylene glycol), poly(oxyethylated glycerol),
poly(oxyethylated sorbitol), poly(oxyethylated
glucose), poly(oxazoline), poly(acryloylmorpholine),
and poly(vinylpyrrolidone); wherein C is carbon;
wherein P and Q comprise linkage fragments that may be
20 the same or different and join polymeric arms poly_a and
poly_b, respectively, to C by hydrolytically stable
linkages selected from the group consisting of amide,
amine, ether, carbamate, thiourea, urea, thiocarbamate,
thiocarbonate, thioether, thioester, and
25 dithiocarbamate linkages; wherein R is a moiety
selected from the group consisting of H, substantially
nonreactive moieties, and linkage fragments having
attached thereto by a hydrolytically stable linkage in
the absence of aromatic rings one or more nonpeptidic

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and substantially nonreactive water soluble polymeric arms; and wherein Z comprises a moiety selected from the group consisting of carboxyl, hydroxyl, trifluoroethylsulfonate, isocyanate, isothiocyanate, 5 N-hydroxylsuccinimidyl ester, N-hydroxylsuccinimidyl carbonate, p-nitrophenylcarbonate, trichlorophenylcarbonate, aldehyde, vinylsulfone, maleimide, iodoacetamide, and iminoesters.

19. A multi-armed monofunctional polymeric 10 derivative that is the reaction product of at least one monofunctional nonpeptidic polymer derivative and a linker moiety having two or more active sites that form linkages with said monofunctional nonpeptidic polymer derivatives in the absence of aromatic moieties, 15 wherein said linkages between said linker moiety and said monofunctional nonpeptidic polymer derivatives are hydrolytically stable.

20. The multi-armed monofunctional polymeric derivative of Claim 19 wherein said linker moiety is selected from the group consisting of monohydroxy 20 alcohols and monocarboxylic acids.

21. The multi-armed monofunctional polymer derivative of Claim 19 wherein said active sites on said linker moiety are nucleophilic moieties.

25 22. The multi-armed monofunctional polymer derivative of Claim 21 wherein said nucleophilic moieties are selected from the group consisting of amino, thiol, and hydroxyl moieties.

30 23. The multi-armed monofunctional polymer derivative of Claim 19 wherein said active sites on said linker moiety are electrophilic moieties.

24. The multi-armed monofunctional polymer derivative of Claim 23 wherein said electrophilic moieties are selected from the group consisting of trifluoroethylsulfonate, isocyanate, isothiocyanate, 5 active esters, active carbonates, aldehyde, vinylsulfone, maleimide, iodoacetamide, and iminoesters.

25. The multi-armed monofunctional polymeric derivative of Claim 24 wherein said active esters are 10 N-hydroxylsuccinimidyl ester and said active carbonates are selected from the group consisting of N-hydroxylsuccinimidyl carbonates, *p*-nitrophenylcarbonates, and trichlorophenylcarbonates.

26. The multi-armed monofunctional polymeric derivative of Claim 19 wherein said hydrolytically 15 stable linkages in the absence of aromatic rings are selected from the group consisting of amide, amine, ether, carbamate, thiourea, urea, thiocarbamate, thiocarbonate, thioether, thioester, and 20 dithiocarbamate linkages.

27. The multi-armed monofunctional polymeric derivative of Claim 19 wherein said monofunctionality is selected from the group consisting of carboxyl, 25 hydroxyl, activated carboxyl, activated hydroxyl, and conjugates of activated carboxyl or hydroxyl sites and molecules having at least one reactive nucleophilic moiety.

28. The multi-armed monofunctional polymeric derivative of Claim 19 wherein said monofunctionality 30 is selected from the group consisting of trifluoroethylsulfonate, isocyanate, isothiocyanate, active esters, active carbonates, aldehyde,

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vinylsulfone, maleimide, iodoacetamide, and iminoesters.

29. The multi-armed monofunctional polymeric derivative of Claim 28 wherein said active ester is N-5 hydroxylsuccinimide and said active carbonates are selected from the group consisting of N-hydroxylsuccinimide carbonates, p-nitrophenylcarbonates, and trichlorophenylcarbonates.

30. The multi-armed monofunctional polymeric derivative of Claim 19 wherein said nonpeptidic polymeric derivative is selected from the group consisting of poly(alkylene oxides), poly(oxyethylated polyols), and poly(oxyethylated glucose).

31. The multi-armed monofunctional polymeric derivative of Claim 19 wherein said nonpeptidic polymer derivative is selected from the group consisting of activated poly(ethylene glycol), poly(vinyl alcohol), poly(propylene glycol), poly(oxyethylated glycerol), poly(oxyethylated sorbitol), poly(oxyethylated glucose), poly(oxazoline), poly(acryloylmorpholine), and poly(vinylpyrrolidone).

32. The multi-armed monofunctional polymeric derivative of Claim 19 wherein said nonpeptidic polymer derivative is a linear mPEG of molecular weight of from 25 about 50 to 50,000 and the multi-armed monofunctional polymeric derivative has two arms of said linear mPEG.

33. A material comprising a solid surface or particle having attached thereto compounds of the structure claimed in Claim 19.

34. The material of Claim 33 wherein said solid surface or particle is a liposome.

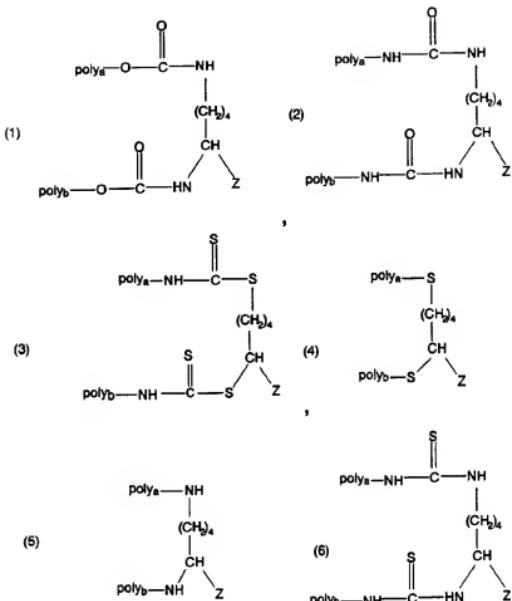
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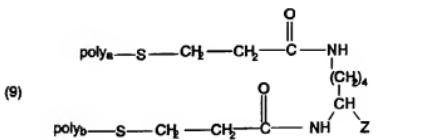
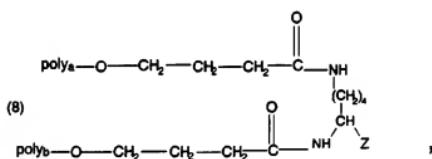
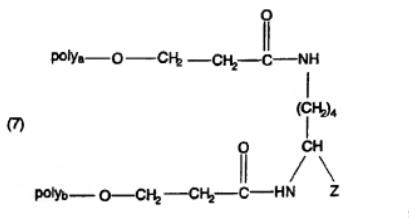
35. A biologically active structure comprising a biologically active molecule having attached thereto one or more compounds of the structure claimed in Claim 19.

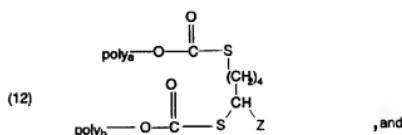
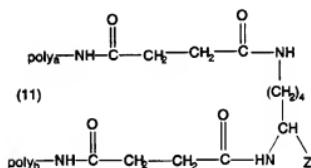
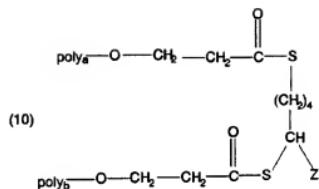
5 36. The biologically active structure of Claim 35 wherein said biologically active molecule is selected from the group consisting of polypeptides, polynucleotides, and lipids.

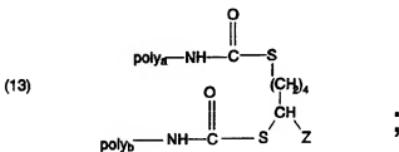
10 37. The biologically active structure of Claim 36 wherein said polypeptide is selected from the group consisting of asparaginase, catalase, ribonuclease, subtilisine, trypsin, and uricase.

15 38. A two-armed polymeric derivative having a structure selected from the group consisting of:









wherein poly_a and poly_b may be the same or different and comprise moieties selected from the group consisting of poly(ethylene glycol), poly(vinyl alcohol), poly(propylene glycol), poly(oxyethylated glycerol),

5 poly(oxyethylated sorbitol), poly(oxyethylated glucose), poly(oxazoline), poly(acryloylmorpholine), and poly(vinylpyrrolidone) moieties; and wherein Z comprises a moiety selected from the group consisting of moieties having a single site reactive toward
10 nucleophilic moieties, sites that can be converted to sites reactive toward nucleophilic moieties, and the reaction product of a nucleophilic moiety and moieties having a single site reactive toward nucleophilic moieties.

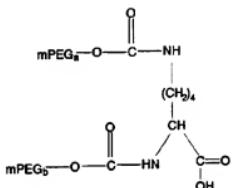
15 39. The two-armed polymeric derivative of
Claim 38 wherein said reactive site is selected from
the group consisting of carboxyl, activated carboxyl,
hydroxyl, activated hydroxyl, and conjugates of
activated carboxyl or hydroxyl sites and molecules
20 having at least one reactive nucleophilic moiety.

40. The polymeric derivative of Claim 38 wherein Z comprises a moiety selected from the group consisting of trifluoroethylsulfonate, isocyanate, isothiocyanate, active esters, active carbonates, 25 aldehyde, vinylsulfone, maleimide, iodoacetamide, and iminoesters.

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41. The polymeric derivative of Claim 40 wherein said active ester is N-hydroxylsuccinimidyl ester and said active carbonates are selected from the group consisting of N-hydroxylsuccinimidyl carbonate, 5 *p*-nitrophenylcarbonate, and trichlorophenylcarbonate.

42. A molecule having the structure



wherein mPEG_a and mPEG_b have the structure CH₃-(CH₂CH₂O)_nCH₂CH₂-, wherein n equals from 1 to about 1,150, and wherein n may be the same or different for 10 mPEG_a and mPEG_b.

43. The molecule of Claim 42 wherein n equals from 1 to about 570.

44. A method of synthesizing a multi-armed, water soluble, monofunctional polymeric molecule 15 comprising reacting one or more nonpeptidic monofunctional polymers of the structure poly-W, wherein W is an active moiety providing the monofunctionality for the polymer, with a linker moiety having two or more active sites with which W is 20 reactive, and forming hydrolytically stable linkages in the absence of aromatic rings between the monofunctional polymer and the linker moiety at the linker moiety active sites, the linker moiety having a reactive site for which said active moiety -W is not

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reactive to provide the monofunctionality for the multi-armed molecule.

45. The method of Claim 44 wherein the
5 method further comprises activating the reactive site
after the multi-armed polymeric compound is formed with
an electrophilic moiety.

46. The method of Claim 45 wherein the
electrophilic moiety is reactive with nucleophilic
10 moieties selected from the group consisting of amino,
thiol, and hydroxyl moieties.

47. The method of Claim 44 wherein the
active moiety W is an electrophilic moiety selected
from the group consisting of trifluoroethylsulfonate,
15 isocyanate, isothiocyanate, active esters, active
carbonates, aldehyde, vinylsulfone, maleimide,
iodacetamide, and iminoesters.

48. The method of Claim 47 wherein the
active ester is N-hydroxylsuccinimidyl ester and the
20 active carbonates are selected from the group
consisting of N-hydroxylsuccinimidyl carbonate,
p-nitrophenylcarbonate, and trichlorophenylcarbonate.

49. The method of Claim 44 wherein the
active moiety W is a nucleophilic moiety selected from
25 the group consisting of amino, thiol, and hydroxyl
moieties.

50. The method of Claim 44 wherein the
active sites on the linker moiety are nucleophilic
moieties selected from the group consisting of amino,
30 thiol, and hydroxyl moieties.

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51. The method of Claim 44 wherein the active sites on the linker moiety are electrophilic moieties selected from the group consisting of trifluoroethylsulfonate, isocyanate, isothiocyanate, 5 active esters, active carbonates, aldehyde, vinylsulfone, maleimide, iodoacetamide, and iminoesters.

52. The method of Claim 51 wherein the active ester is N-hydroxylsuccinimidyl ester and the 10 active carbonates are selected from the group consisting of N-hydroxylsuccinimidyl carbonate, *p*-nitrophenylcarbonate, and trichlorophenylcarbonate.

53. The method of Claim 44 wherein the hydrolytically stable linkages are selected from the 15 group consisting of amide, amine, ether, carbamate, thiourea, urea, thiocarbamate, thiocarbonate, thioether, thioester, and dithiocarbamate linkages.

54. A method for preparing a polymeric derivative represented by the structure



20 comprising the steps of:

a) reacting nonpeptidic, water soluble, monofunctional polymers of the structure $\text{poly}_a\text{-W}$ and $\text{poly}_b\text{-W}$ with a linker moiety having at least two active sites for which W is selective, a reactive site Z for 25 which W is not selective, and a moiety R which is substantially nonreactive, wherein W is an active electrophilic moiety selected from the group consisting of trifluoroethylsulfonate, isocyanate, isothiocyanate, active esters, active carbonates, aldehyde,

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vinylsulfone, maleimide, iodoacetamide, and iminoesters, and may be the same or different on poly_a and poly_b, wherein poly_a and poly_b are polymer moieties selected from the group consisting of poly(ethylene glycol), poly(vinyl alcohol), poly(propylene glycol), poly(oxyethylated glycerol), poly(oxyethylated sorbitol), poly(oxyethylated glucose), poly(oxazoline), poly(acryloylmorpholine), and poly(vinylpyrrolidone) and may be the same or different, and wherein the active sites of the linker moiety are nucleophilic sites selected from the group consisting of amino, thiol, and hydroxyl; and

b) forming hydrolytically stable linkages P and Q, which may be the same or different, in the absence of aromatic rings between the polymer and the linker moiety that are selected from the group consisting of amide, amine, ether, carbamate, thiourea, urea, thiocarbamate, thiocarbonate, thioether, thioester, and dithiocarbamate linkages.

20 55. The method of Claim 54 wherein the linker moiety is substituted with polymer at each active site in one step.

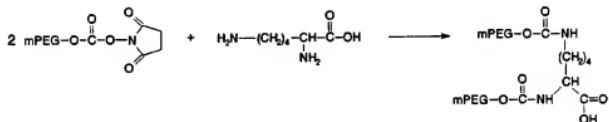
25 56. The method of Claim 55 wherein the linker moiety is substituted with polymer at each active site in more than one step.

57. The multi-armed polymeric derivative of Claim 54 wherein said linker moiety is selected from the group consisting of monohydroxy alcohols and monocarboxilic acids having two or more active moieties selected from the group consisting of thiol, amino, and hydroxyl moieties.

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58. The multi-armed polymeric derivative of
Claim 1 wherein Z is selected from the group consisting
of carboxyl, hydroxyl, activated carboxyl, activated
hydroxyl, and conjugates of precursor activated
5 carboxyl or hydroxyl sites and molecules having sites
for which said precursor activated sites are active.

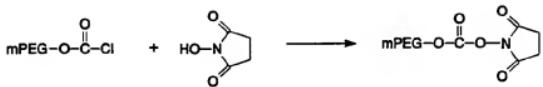
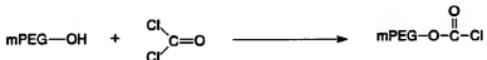
59. A method for forming monofunctional
monomethoxy-poly(ethylene glycol) disubstituted lysene
comprising the following step:



10 60. The method of Claim 59 wherein the
reaction takes place in water at a pH of about 8.0.

61. The method of Claim 59 further
comprising the steps of

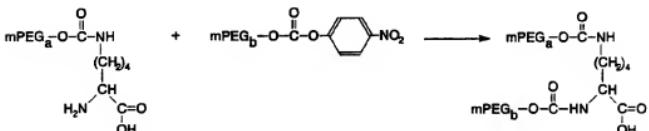
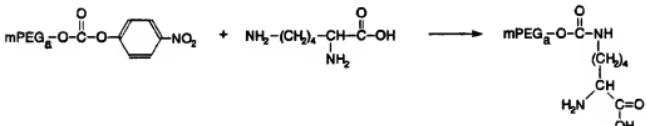
- 85 -



62. The method of Claim 61 wherein steps a) and b) take place in methylene chloride.

63. The method of Claim 59 further comprising the steps of activating the carboxyl moiety 5 and reacting the activated carboxyl moiety with an active moiety to join the disubstituted lysine to the active moiety.

64. A method for forming a monofunctional monomethoxy-poly(ethylene glycol) disubstituted 10 compound comprising the following steps:



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65. The method of Claim 64 further comprising the steps of activating the carboxyl moiety and reacting the activated carboxyl moiety with an active moiety to join the disubstituted lysine to the 5 active moiety.

66. The method of Claim 64 wherein step a) takes place in aqueous buffer.

67. The method of Claim 64 wherein step b) takes place in methylene chloride.

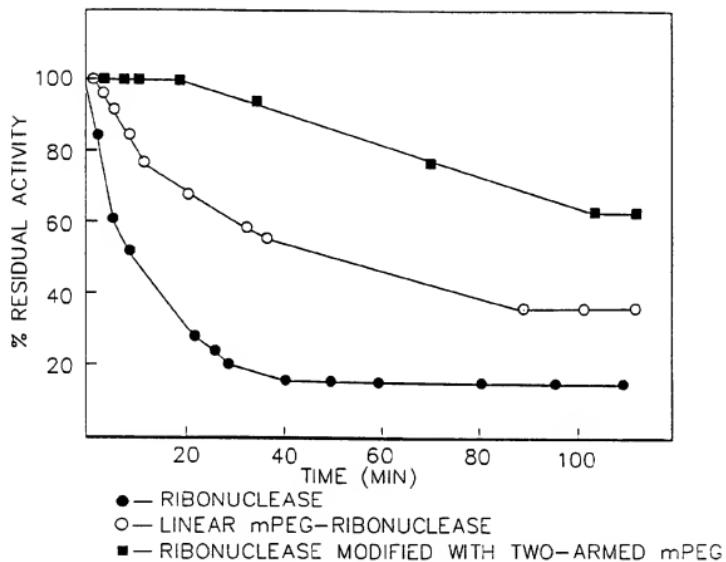


FIG. 1a.

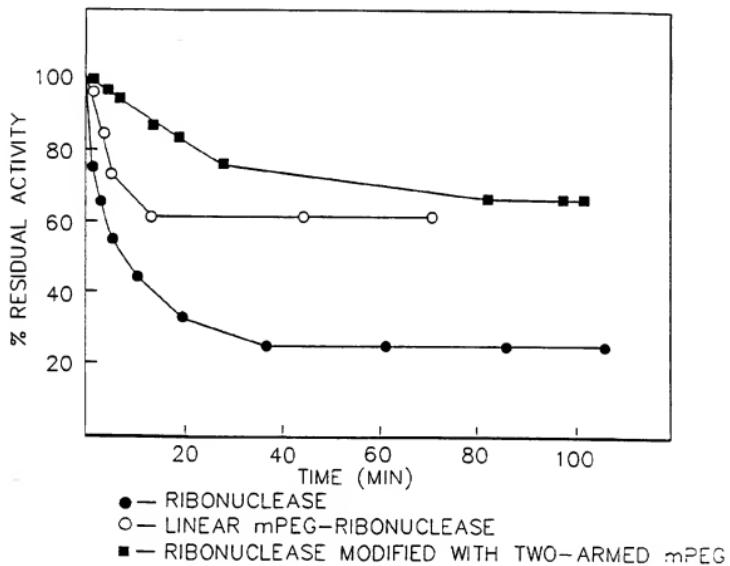


FIG. 1b.

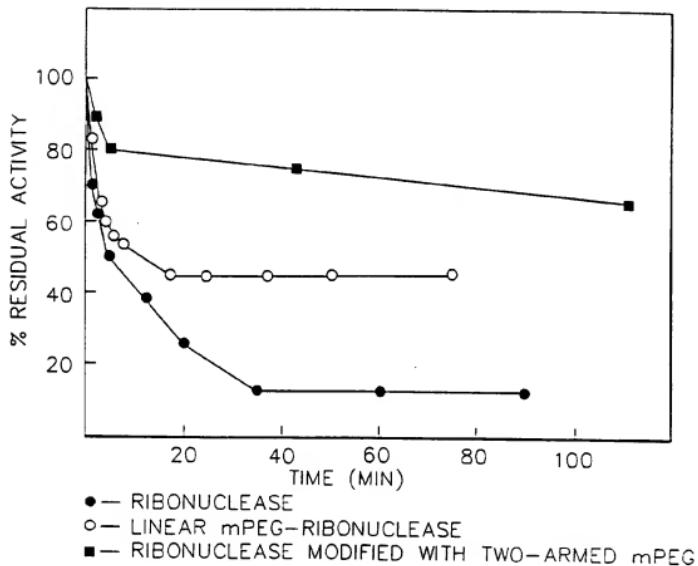
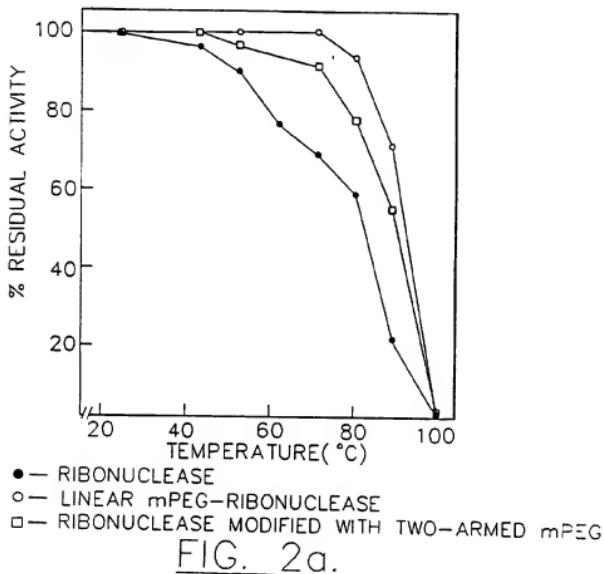


FIG. 1c.



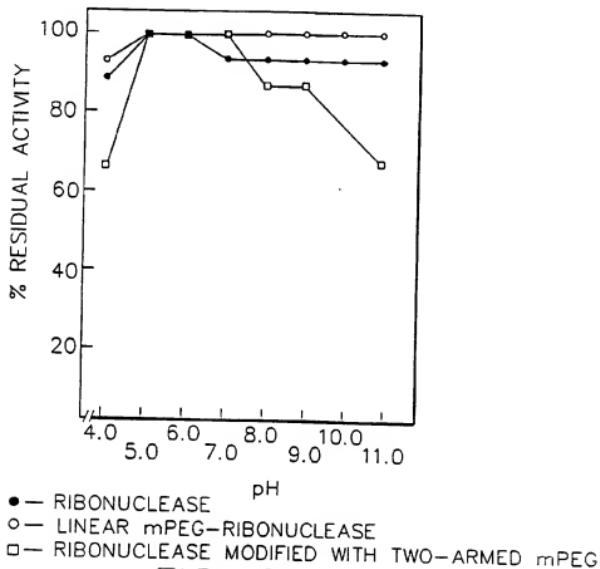


FIG. 2b.

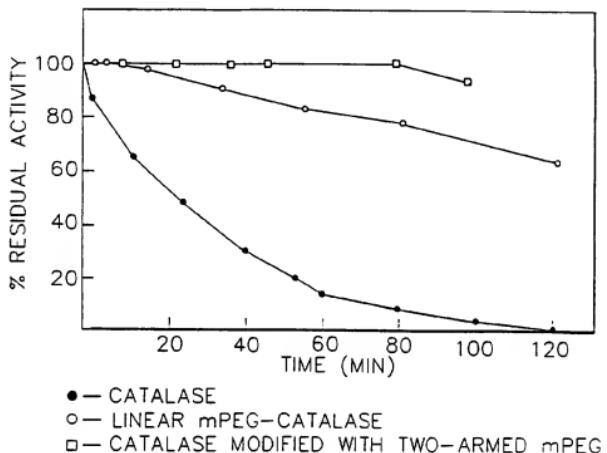


FIG. 3a.

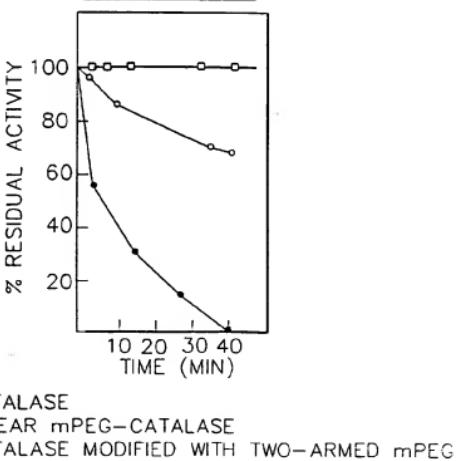


FIG. 3b.

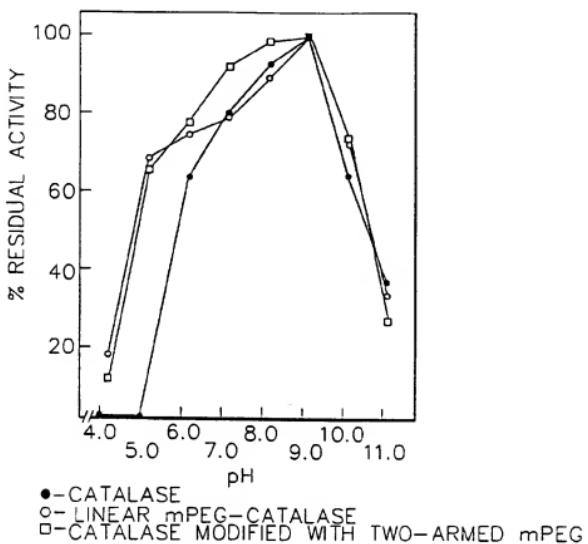


FIG. 4.

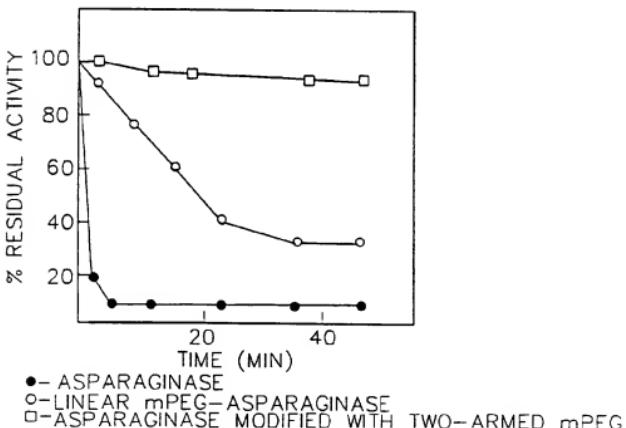


FIG. 5.

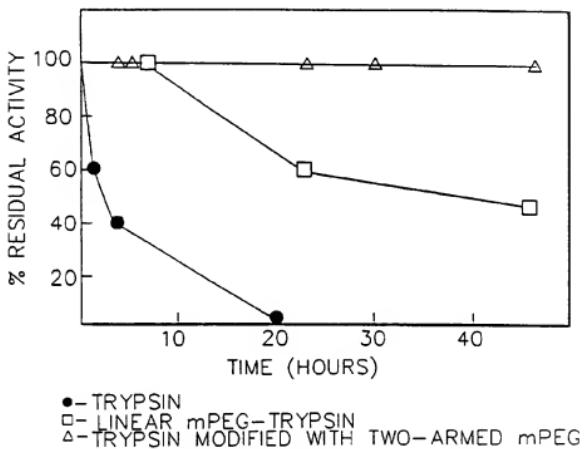


FIG. 6.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/00474A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48 C08G55/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C08G A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	BIOCONJUGATE CHEMISTRY, vol. 06, no. 01, January 1995, WASHINGTON D.C., pages 62-69, XP002004192 MONFARDINI C. ET AL: "A branched monomethoxypolyethyleneglycol for protein modifications" *Whole document* --- WO,A,95 11924 (ENZON INC.) 4 May 1995 see examples 1-8 --- -/-	1-66
P, X		1-5, 8-19, 30-32, 35,36, 38,39,55

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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"A" document member of the same patent family

Date of the actual completion of the international search 29 May 1996	Date of mailing of the international search report 10.06.96
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O'Sullivan, T

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/US 96/00474

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 400 486 (SUMITOMO PHARMACEUTICALS CO.) 5 December 1990 see claim 1 ---	1-66
A	EP,A,0 400 472 (SUMITOMO PHARMACEUTICALS CO.) 5 December 1990 see claims 1,22 ---	1-66
A	EP,A,0 632 082 (HEYLECINA S.A.) 4 January 1995 see page 11, line 1 - line 25 -----	1-66

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ink onal Application No

PCT/US 96/00474

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9511924	04-05-95	AU-B- 8090294	22-05-95
EP-A-0400486	05-12-90	JP-A- 3088822	15-04-91
EP-A-0400472	05-12-90	AT-T- 136315 CA-A- 2017543 DE-D- 69026306 JP-A- 3095200 US-A- 5342940 JP-A- 3072469	15-04-96 27-11-90 09-05-96 19-04-91 30-08-94 27-03-91
EP-A-0632082	04-01-95	NONE	